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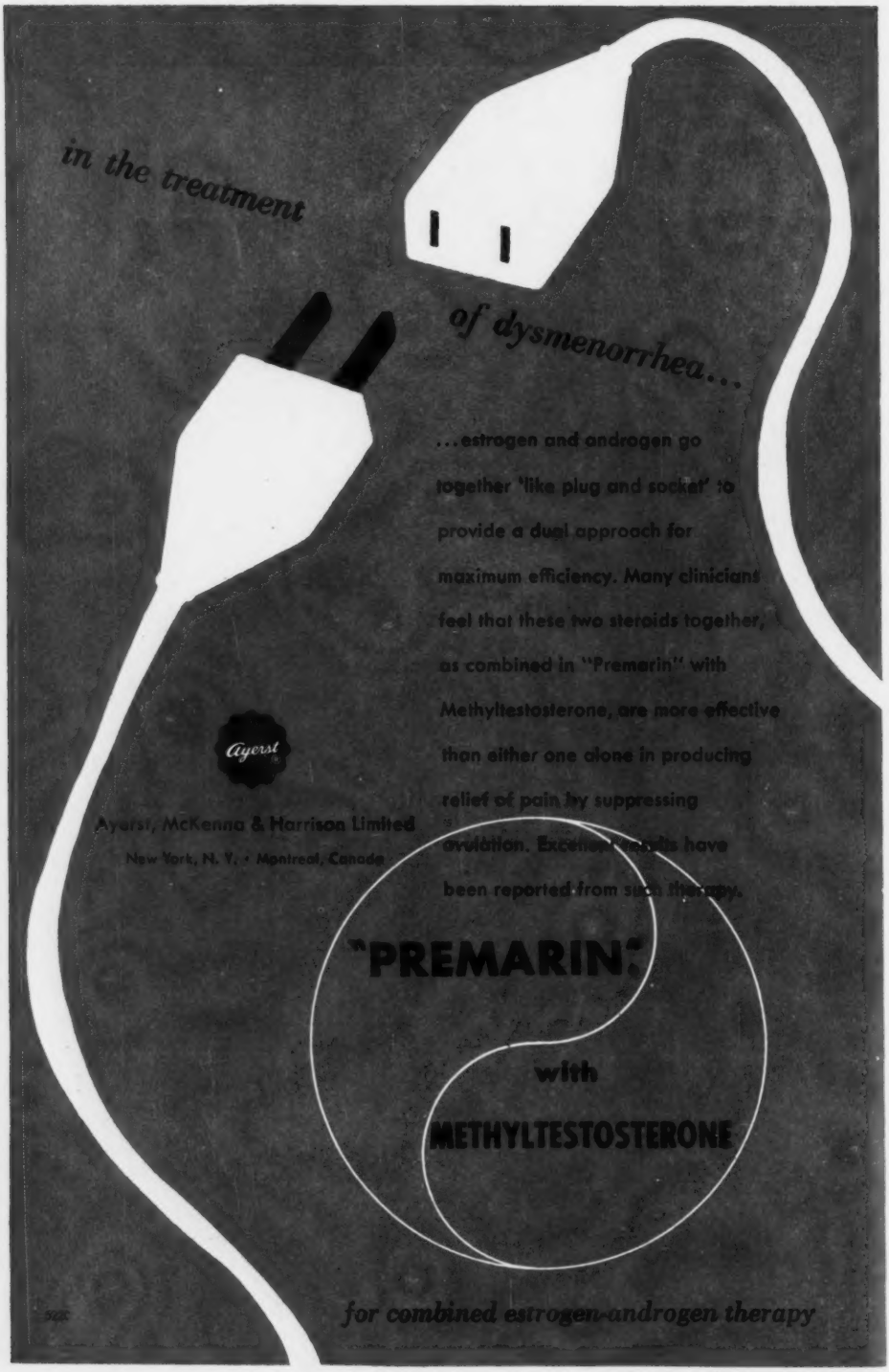
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VOLUME 50 JUNE, 1952 NUMBER 6

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THE FATE OF C^{14}-TESTOSTERONE AND C^{14}-PROGESTERONE IN MICE AND RATS[1,2,3]

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THIS investigation of the tissue distribution and metabolic fate of radioactive testosterone and progesterone in mice and rats was undertaken with the primary aim of determining by animal experimentation whether the administration of a hormone containing C^{14} would be a serious hazard to the animal and secondarily, with the hope that experiments with rodents might provide information helpful in the study of steroid hormone metabolism in man. These considerations restricted the experimental approach to a survey rather than a detailed analysis since our principal interest was the application of tracer studies to human steroid metabolism. The major portion of the experimental work was done therefore with testosterone since the fate of this hormone in humans has been more thoroughly investigated (Dorfman, 1948; Dobriner and Lieberman, 1950). The results obtained provide a decisive answer to the primary question since it is clear that the male hormone, testosterone, and the female hormone, progesterone, remain such a short time in the rodent that the radiation from the isotope does not constitute any reasonable

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[1] A portion of the results embodied in this report were summarized at the Laurentian Hormone Conference, September 1950 (Gallagher, Fukushima, Barry and Dobriner, 1951).

[2] We are deeply grateful to Doctor R. B. Turner who prepared the radioactive testosterone (Turner, 1950) and to Doctor H. B. MacPhillamy who prepared the radioactive progesterone (MacPhillamy and Scholz, 1949).

[3] This investigation was supported by grants from the Lillia Babbitt Hyde Foundation, the National Cancer Institute, United States Public Health Service and the Anna Fuller Fund.

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threat to the animal. Moreover, the hormone and its metabolites were not significantly accumulated by any of the organs or tissue examined and, therefore, damage to a particular target area would not be expected. The steroid hormones thus are not stored in the gland like the thyroid hormone and testosterone has a short biological half-life in contrast to a sterol like cholesterol. On the basis of these studies C^{14} labeled testosterone is a relatively innocuous substance in rodents.

EXPERIMENTAL

Measurement of radioactivity: (a) Gas counting. This technique was employed primarily for the analysis of respiratory carbon dioxide and for whole organs where low activity was anticipated. Samples were oxidized according to the Van Slyke and Folch (1940) procedure, the carbon dioxide was absorbed in sodium hydroxide and was precipitated as barium carbonate. The barium carbonate was transferred to an appropriate vessel in a vacuum line and was reconverted to carbon dioxide with perchloric acid; the activity of the carbon dioxide was determined by internal gas counting using a 16 mm. I.D. counting tube described by Eidinoff (1950). The counting gas mixture consisted of 50 cm. carbon dioxide admixed with 2 cm. carbon disulfide (Miller, 1947). The counter tube was operated in the Geiger-Müller region at approx. 4000 volts and had a plateau of over 200 volts with slopes less than 2 per cent per hundred volts. (b) Direct Plating. Solutions such as urine and alcohol extracts of tissues were evaporated on 10 sq. cm. cupped aluminum planchets so that amounts of material less than 0.5 mg. per sq. cm. of surface were obtained. Self-absorption corrections were neglected. Measurements were made in a windowless flow-gas counter (Radiation Counter Laboratories, Mark 12 Model 1). Appropriate intercalibration data were obtained for the two methods; results are, for the most part, given as counts per minute (c/m) which correspond to $\frac{2}{3}$ of the total disintegrations per minute.

Excretion of radioactivity by mice in the first 72 hours following a single administration of progesterone or testosterone: Three mice were injected intraperitoneally with 0.5 ml. of 30 per cent aqueous propylene glycol containing 86 μg. of 21-C^{14}-progesterone (230,000 disintegrations per minute); three mice were similarly treated with 0.5 ml. of 20 per cent aqueous propylene glycol containing 113 μg. of 4-C^{14}-testosterone (90,000 disintegrations

TABLE I. RECOVERY OF RADIOACTIVITY AFTER A SINGLE INTRAPERITONEAL DOSE OF PROGESTERONE AND TESTOSTERONE IN MICE

Animal		Radioactivity in per cent of administered dose				
		Respiratory CO_2			Urine and feces	Total recovery
		0–24 hrs.	24–48 hrs.	48–72 hrs.		
M-3	86 μg Progesterone	8.6	0.4	0.2	72	81
M-4	86 μg Progesterone	8.8	0.5	0.8	62	72
M-5	86 μg Progesterone	7.5	0.3	0.3	75	83
M-6	113 μg Testosterone	0	0	0	96	96
M-7	113 μg Testosterone	0	0	0	86	86
M-8	113 μg Testosterone	0	0	0	101	101

The mice weighted between 23 and 26 grams.
Mice 3, 4, 6 & 7 were CFW-strain, 5 & 8 were AKM strain.

TABLE 2. DISTRIBUTION OF RADIOACTIVITY IN EXCRETA OF MICE AND RATS AFTER A SINGLE INTRAPERITONEAL INJECTION OF TESTOSTERONE RADIOACTIVITY IN PER CENT OF ADMINISTERED DOSE

Animal	Testosterone μg	Collection period hours	Urine	Feces		Total recovery
				Alcohol soluble	Alcohol insoluble	
M- 9 ♂	113	72	39	44	8	91
M-10 ♂	113	72	34	40	11	85
M-11 ♀	113	72	49	31	12	92
M-12 ♂	113	24	32	52	13	97
M-13 ♂	113	24	47	45	13	105
M-14 ♂	113	24	43	47	11	101
M-16 ♂	5.4	24	36	50	11	97
M-17 ♂	5.4	24	42	42	2	86
M-18 ♂	226	24	29	52	9	90
M-19 ♂	121	24	70	17	1	88
M-20 ♂	121	24	35	42	4	81
R - 3 ♂	124	24	7	40	19	66
R - 4 ♂	9.8	24	12	31	1	44
R - 5 ♂	242	24				54

M = Mouse. R = Rat.

All mice except 19 and 20 were CFW strain, weighing from 22 to 24 gm.; weight of M-18 is 29 gm. 19 and 20 were BAF_1 strain.

Each weighed 33 gm. The three rats were RF and weighed about 240 gm.

per minute). The animals were maintained separately in a glass desiccator for 3 days with a flow of carbon dioxide free air and access to food and water. The exit gases were bubbled through a tower of 10 per cent NaOH. The urine and feces were not separately collected; the combined excreta were continuously extracted with ethanol for 48 hours and portions of the solution were oxidized and counted as CO_2. The solid residue remaining after extraction of the excreta was homogenized and portions were oxidized and counted as CO_2. The results are presented in Table 1.

Excretion of radioactivity in the urine and feces of mice and rats at intervals following variable amounts of testosterone: These studies were made following a single intraperitoneal injection of 4-C^{14}-testosterone in 20 per cent aqueous propylene glycol. The animals were kept in glass metabolism cages and urine and feces were separately collected. Food and water were available to the mice; water only was supplied to the rats. Feces, as collected, were extracted continuously for 48 hours with ethanol using a porous thimble attached to the condenser; the extracted residue was pulverized in a mortar and samples were oxidized. The ethanol extracts were for the most part analyzed by direct plating; portions of the residues were oxidized and counted as CO_2. The urines collected were diluted with the water used to wash the cage and the radioactivity was determined by direct plating except the urines from M-16 and M-17 which were oxidized and counted as CO_2. The results are presented in Table 2.

Distribution of radioactivity in tissues following a single intraperitoneal injection of testosterone: The tissues of three normal mice, a castrate mouse, a normal rat and a mouse with a transplantable mammary adenocarcinoma were examined for radioactivity. 4-C^{14} testosterone in the amounts shown in Tables 3, 4, 5 and 6 was injected intraperitoneally in 20 per cent aqueous propylene glycol. The animals were sacrificed at the times indicated. The tissues were dissected, weighed and dried in a vacuum at room temperature. Feces, livers and the various portions of the gastro-intestinal tract were ground in

TABLE 3. RAT (R-5) DISTRIBUTION OF RADIOACTIVITY 24 HOURS AFTER 242 μg. (129,000 COUNTS PER MINUTE) OF TESTOSTERONE INTRAPERITONEALLY

	Recovered radioactivity	
	Counts per minute	Per cent of dose
Urine and Feces	70,000	54
Colon and Contents	40,000	31
Ileum and Contents	8,600	6.7
Jejunem, Duodenum and Contents	1,440	1.1
Stomach	160	0.1
Liver	1,000	0.8
Kidneys	160	0.1
Total	121,000	94.

The following tissues contained no measurable C^{14}: Spleen, Seminal Vesicles, Testes and Epididymes, Adrenals, Heart and Brain.

a mortar and after drying were extracted continuously with ethanol for 48 hours. The ethanol extracts were counted by direct plating. The large intestine of mouse M-15 was opened and the contents were washed into a beaker with water. The washed tissue was dried, powdered and extracted continuously with ethanol; the intestinal contents and washings were evaporated on a steam bath and combined with the feces. The gall bladder and contents of M-15 were extracted continuously with ethanol and the extract counted by direct plating. Blood from M-21 and M-22 was drawn into a beaker moistened with heparin; the blood volume was assumed to be 3 ml. The remaining dried tissues were oxidized in their entirety and radioactivity was determined by gas counting. The results are recorded in Tables 3, 4, 5 and 6.

Fractionation of excreted radioactivity: (*a*) *Urine.* A 24 hours urine collection from mouse M-18 (226 μg. of testosterone) was diluted to 100 ml. with water and the solution was stored overnight in the refrigerator. A 40 ml. portion (350 c/m) was acidified with 10 ml. of 9 N. sulfuric acid and heated under a reflux for 30 minutes. After cooling, the hydrolyzed urine was diluted to 500 ml. with water and extracted continuously with

TABLE 4. MALE MOUSE (M-15). DISTRIBUTION OF RADIOACTIVITY 4¼ HOURS AFTER 113 μg (59,800 C/M) OF TESTOSTERONE INTRAPERITONEALLY

	Recovered radioactivity	
	Counts per minute	Per cent of dose
Urine	19,000	32
Feces and Colon Contents	19,800	33
Colon (washed)	1,200	2
Intestine and Contents	6,800	11
Stomach	75	0.13
Liver	2,800	4.7
Gall Bladder	4,600	7.7
Kidneys	71	0.12
Total	54,500	91

Tissues examined which contained less than 0.1% of the dose administered together with the total counts per minute in the organ were: Spleen (20), seminal vesicles (17), testes and epididymes (46), heart (9), lungs (18), bones of the hind limbs (16), Brain (0), adrenals (0).

TABLE 5. NORMAL AND CASTRATE MOUSE. DISTRIBUTION OF RADIOACTIVITY 24 HOURS AFTER TESTOSTERONE INTRAPERITONEALLY

	Normal Mouse (M-12) 113 μg. testosterone (59,800 c/m)		Castrate Mouse (M-19)* 121 μg. testosterone (72,200 c/m)	
	c/m	Per cent of dose	c/m	Per cent of dose
Urine	19,100	32	50,800	70
Feces	38,800	65	13,200	18
Total		97		88

M-12 was CFW strain; M-19 was BAF_1 strain.

Organs examined which contained less than 0.1 per cent of dose administered and the counts per minute in each organ were:

(A). Normal animal: Seminal Vesicles, testes and epididymes, adrenals, heart, lung, kidneys and brain were all negative.

(B). Castrate animal: Spleen (4), seminal vesicles (35), adrenals (19), pancreas (12), thyroid (9), gall bladder (17), pituitary (15), brain, thymus submaxillary gland, heart, lungs were negative.

* Age 10 months, castrated at 5 weeks, weight 33 grams.

TABLE 6. EXCRETION AND TISSUE DISTRIBUTION 65 MINUTES AFTER 60 μG. (36,100 C/M) OF TESTOSTERONE INTRAPERITONEALLY IN A NORMAL C_3H MOUSE AND A C_3H MOUSE WITH A 725 MG. TRANSPLANTED MAMMARY ADRENOCARCINOMA. C_3HBA

Tissue	Radio activity			
	Normal		Tumor	
	c/m	Per cent of dose	c/m	Per cent of dose
Urine	4,500	12	3,700	10
G.I. Tract and contents (alcohol extract)	20,000	55	22,500	62
Liver	3,900	11	4,500	12
Gall bladder	450	1.2	280	0.8
Blood	450	1.2	350	1.0
Bladder	150	0.4	10	0.03
Kidneys	180	0.5	250	0.7
G.I. Tract residue after alcohol extraction	110	0.3	145	0.4
Seminal Vesicles	33	0.09		0.05
Testes	28	0.08	31	0.09
Pancreas	198	0.5	15	0.04
Spleen	35	0.1	35	0.1
Heart	26	0.07	18	.05
Lungs	45	0.1	47	0.1
Thymus	9	0.02	4	0.01
Submaxillary gland	20	0.06	12	0.03
Brain	7	0.02	5	0.01
Tumor			150	0.4
Tumor residue			0	0.0
Preputial glands	0	0.0	9	0.02
Total		82.64		89.83

In the normal animal, preputial glands, adrenals, thyroid and pituitary gland were negative.

In the tumor animal, adrenals, thyroid and pituitary gland were negative.

ether for 48 hours. The ether extract, 300 ml. volume, was extracted five times, each with 50 ml. of 5 per cent sodium chloride solution and the aqueous washes were each re-extracted twice with 200 ml. of ether. The combined ether solutions contained 41 c/m (12 per cent of the total activity).

The sodium chloride extracts were combined and 15 g of sodium chloride were added to the approximately 300 ml. of solution. The brine solution was then extracted five times with 300 ml. portions of *n*-butanol. The butanol extracts were combined and contained 81 c/m (23 per cent of the total activity).

The dilute urine after the continuous ether extraction was extracted five times with separate 500 ml. portions *n*-butanol after the addition of 50 g of sodium chloride. The combined butanol extracts contained 168 c/m (48 per cent of the total activity). The results are charted in Fig. 1.

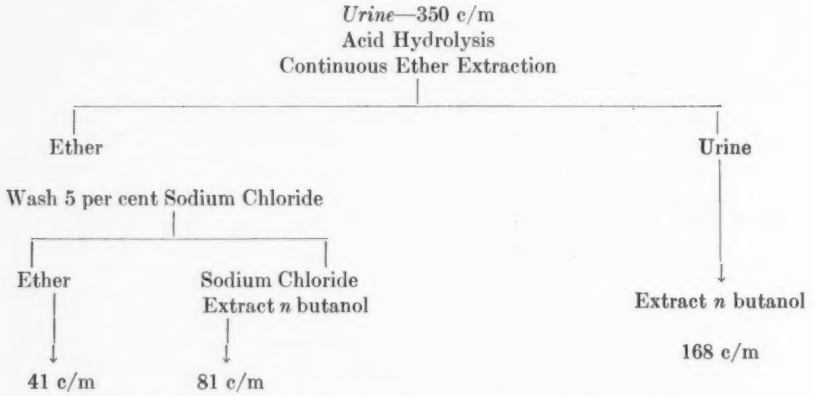

FIG. 1. Flow diagram of fractionation of mouse urinary radioactivity after testosterone C^{14} administration (mouse 18).

Another 40 ml. portion (350 c/m) of the same urine was made to a 1 N. sodium hydroxide solution and heated under reflux for 30 minutes. Continuous ether extraction for 48 hours of the alkaline solution yielded 18 c/m in the ether extract (5 per cent of the total activity).

After administration of 113 μg. of 4-C^{14}-testosterone, seventy two hour urine collections from each of two mice (M-10, M-11) were submitted to the same procedures. The urines were diluted to 100 ml. with water and stored in the refrigerator. 25 ml. of the diluted urine was added to 15 ml. of water and 2 ml. of conc. sulfuric acid and the mixture was heated under reflux for 2 hours. After cooling the aqueous solution was extracted in a separatory funnel four times with fresh 40 ml. portions of ether. The combined ether extracts were washed twice with 75 ml. portions of 5 per cent sulfuric acid and twice with 50 ml. portions of water. After drying over sodium sulfate the ether was removed and the ether soluble material contained 12 per cent of the total activity in one experiment and 23 per cent in the other.

All the aqueous solutions were combined, 30 g of sodium chloride were added and the solution was extracted five times with 200 ml. portions of *n*-butanol. The combined butanol extracts, approximately 1.liter, were washed twice with 600 ml. portions of 10 per

cent sodium chloride solution and most of the solvent was removed under diminished pressure. The residue was diluted to 100 ml. with butanol and contained in both experiments 80 per cent of the total radioactivity present in the urine. The aqueous portion was discarded. 25 ml. of the butanol solution was diluted to 50 ml. with *n*-butanol and was extracted five times with fresh 50 ml. portions of 5 per cent sodium hydroxide solution containing 10 per cent sodium chloride. The combined alkaline extracts were re-extracted twice with 200 ml. portions of butanol. The butanol extracts were combined and washed twice with 300 ml. portions of 10 per cent sodium chloride solution and the butanol solution was then concentrated to a small volume under diminished pressure.

The aqueous alkaline washes (approximately 1 liter) were acidified with sulfuric acid, extracted 3 times each with 300 ml. of butanol, the butanol solution was washed twice with 1 liter portions of 10 per cent sodium chloride solution and the solvent was removed. The results are shown in Fig. 2. (*b*) *Feces:* Three day fecal collections from mice, M-10 and M-11, were extracted continuously for 48 hours with ethanol and the ethanol extract made to 100 ml. volume. A 10 ml. portion was diluted with 210 ml. of water and was distilled at atmospheric pressure until 90 ml. of distillate had been collected. The residual aqueous suspension was acidified with 7.5 ml. of conc. sulfuric acid in 20 ml. of water and was heated under a reflux for 2 hours. The fractionation from that stage was precisely that used for the urine. The results are shown in Fig. 3.

DISCUSSION

In as much as the hormones as a class are not species specific and since testosterone produces essentially the same physiological response in men and mice, it might be anticipated that experiments in rodents would

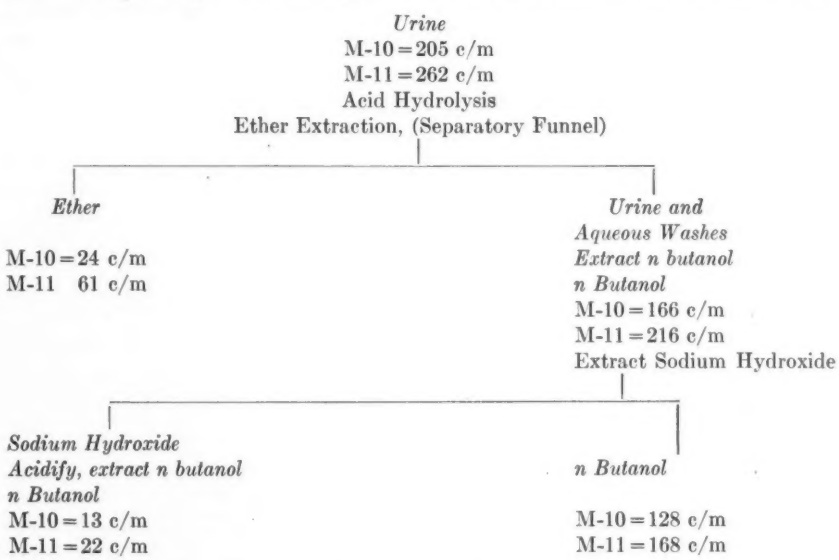

FIG. 2. Flow diagram of fractionation of mouse urinary radioactivity after testosterone-C^{14} administration.

provide data that could be translated to other species including man. Despite the similarity of action, the metabolic transformation and disposal of testosterone by mice and rats is totally different from that of the human. The route of excretion is dissimilar since in man the metabolites are eliminated in the urine whereas in the rodent, more than half the total radioactivity after testosterone was found in the intestinal tract and in the feces. Species variability is to be anticipated, yet even closely related species like mice and rats eliminate the metabolites of testosterone in dissimilar fashion. Thus, with the rat approximately 10 per cent of the radioactivity was found in the urine while mice excreted from 30 to 50 per cent in urine. In the human the major products of testosterone metabolism are the saturated ketones androsterone and etiocholanolone, conjugated with sulfuric acid or with glucuronic acid, while the principal products in the rodent are clearly not these compounds or conjugates. The rodents, therefore, do not provide information that can readily be extended to the human species.

It should be emphasized that these hormones were injected intraperitoneally in aqueous propylene glycol solution. Given in this way absorption of the steroid was complete as evidenced by the rapidity of distribution and excretion while deposition of the administered hormone at the injection site as with oil solutions or aqueous crystalline suspension was avoided.

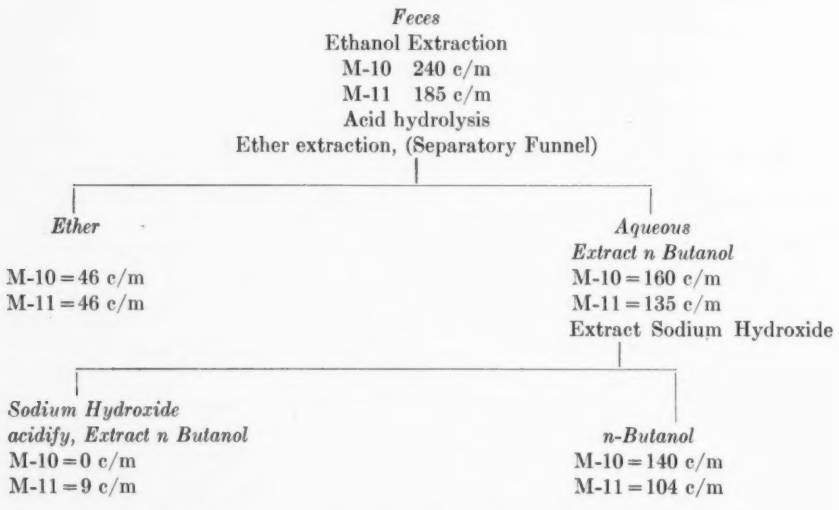

FIG. 3. Flow diagram of fractionation of mouse fecal radioactivity after testosterone C^{14} administration.

However, other routes of administration might lead to different results and therefore the importance of the intraperitoneal site of injection should be clearly in mind in the evaluation of the evidence.

It is extremely important to bear in mind further that radioactivity alone was measured and that this represents what is or was carbon-4 of testosterone or carbon-21 of progesterone and not necessarily, therefore, the hormone itself. In the absence of chemical characterization the mere presence of radioactivity is not evidence for the compound administered.

The excretion of radioactivity occurred promptly and the majority of the hormone was lost to the animal in a short time. Within 65 minutes after injection of testosterone there was less than 15 per cent of the administered radioactivity in tissues other than the gastrointestinal tract and its contents; within 24 hours after testosterone or progesterone, practically all of the administered radioactivity was outside the body. Almost all of the small amount retained in the 65 minute experiment was in the liver, gall bladder and kidneys and was therefore in the process of elimination. The most striking aspect of the experiment, thus, is the very short term the administered hormone is in contact with the tissues. This finding, of course, raised the possibility that the tissues of normal animals are saturated with the hormone or its metabolites so that any additional administered compound is treated as an excess and rapidly excreted. Against this view, several facts may be cited. First it would be anticipated that the administered hormone or its metabolites would exchange freely with the same substance in the tissues. Second, there seemed to be no difference between handling of testosterone by the male or the female animal and since the tissues of the female would scarcely be saturated with testosterone, this is evidence against such an objection. Third and very pertinent, is the fact that no difference was discernible between the fate of 5.4 μg. and 226 μg. It is possible of course that the true hormone is secreted into the blood in a form other than free testosterone and in this event the foregoing remarks are not valid objections. Since, however, there was virtually no radioactivity in the tissues, the hormone was certainly not retained by the animal in appreciable amount. For the present, then, it must be concluded that testosterone in the mouse is not stored in tissues and never reaches a high level in organs other than those concerned with its excretion. This finding provides a decisive answer to the question of radiation hazard following administration of C^{14}-testosterone since the hormone and its metabolites were so promptly excreted. Even after one hour if the total radioactivity retained were concentrated in the liver the amount of radiation from very much larger doses than we have used would be well under permissible limits. Since even this small amount is not stored, the potential danger from the use of this hormone in tracer amount seems negligible.

The implication of these findings for an understanding of hormone action is very significant. The fact that there is only a trace of hormone in tissues a short time after injection can be interpreted as evidence that testosterone is effective in very minute amounts and/or that the biological action is achieved in a very short time interval. If the radioactivity in the tissues is testosterone—a dubious assumption—the amount may be the physiologically active material and the major portion of the small dose actually injected was for the most part wasted by the animal. Alternately, the view can be taken that a larger amount of hormone reaches the circulation for a limited interval of time and this brief burst of hormone stimulus is sufficient to initiate or control by some triggering mechanism the biochemical processes under hormonal control. In either event, tissue localization or deposition would not be expected and this is in accord with the experimental results. Indeed, it might well be argued that the hormone does not remain in the body because it does not enter into reaction as a component of a biochemical system in the sense that a vitamin or a phospholipid exercises a persistent cellular function. On the contrary, the hormone, by its fleeting presence may initiate processes or control the rates of reaction by its physical presence in the medium in much the way that hydrogen ion concentration influences a reaction rate. The biochemical regulation of cellular economy by hormones may thus be dependent upon the continuous circulation of a definite amount of a very specific compound which is in turn metabolized and excreted by some or all tissues quite apart from its regulatory function.

Certain results of these studies illuminate the comparative metabolism of hormones and may thus serve as a working basis for further experiments. Comparison of progesterone metabolism by the rodent and man affords an illustration. During the first 24 hours following the administration to mice of progesterone labeled at C-21, approximately 8 per cent of the radioactivity was found in the CO_2, in substantial agreement with Reigel, Hartrop and Kittinger (1950). The appearance of the C-21 carbon of progesterone in the respiratory CO_2 may offer an explanation for the failure to find significant quantities of the metabolites of progesterone in the human. Pregnanediol and to a lesser extent pregnanolone are products of the biochemical transformation of endogenous or administered progesterone in the human species (Pearlman, 1948; Dobriner and Lieberman, 1950) but these two metabolites account for less than 20 per cent of the administered hormone. Little or none of administered progesterone in humans appears in the C-19 ketosteroid fraction of urine and it is probable ring D of the steroid undergoes fragmentation in the course of this oxidative cleavage of the side chain. Loss of the side chain may result in the elimination of a fragment which then enters the two carbon "acetate"

pool and results in a wide distribution of radioactivity in cellular constituents.

The excretory products of testosterone in mice, for the most part are neither testosterone nor chemically closely related steroids such as androsterone and etiocholanolone, the principal end products of testosterone metabolism in humans. Fractionation of urinary and fecal radioactivity demonstrated that only about 12 per cent of the excretory products were ether soluble i.e. in what might conventionally be considered the steroid fraction. The remainder of the radioactivity was in water soluble material. Whether this water soluble material represents a conjugate of a steroid resistant to hydrolysis by both acid and base or whether the radioactivity is present in a degradation product without an intact steroid nucleus cannot be decided on the available evidence. The similar fractionation of both urinary and fecal material is indicative that a closely related compound or class of compounds was present in both excretions but failing isolation and further characterization this conclusion must be tentative. If the excretion product were a simple derivative of testosterone resulting, for example, from oxidative fission of one or perhaps two of the rings, it is unlikely that the product would be water soluble to such an extent that even continuous ether extraction would fail to remove it from water. Further, assuming such extensive degradation, a portion of the radioactivity might be expected to appear in CO_2 and this was not the case. It is this point that speaks against any considerable rupture of the steroid nucleus, because the isotopic C-4 was in the most highly oxidized portion of the molecule and on purely chemical grounds might be expected to undergo cleavage from the rest of the ring system more readily than any other carbon atom. Since the physical properties of the testosterone metabolite were so markedly changed it might reasonably be concluded that the chemical alteration was extensive. The investigations of Samuels, McCaulay and Sellers (1947) with the transformation of testosterone by rodent liver are pertinent to the fate of carbon-4. They found that the α-β unsaturated ketone system of testosterone rapidly disappeared in the presence of liver homogenates and they were unable to account for the product in terms of ketosteroids measured by the Zimmerman reaction. Both their results and those reported here are in agreement on the rapid transformation of testosterone by the rodent but the chemistry of the transformation is unknown. Various possibilities could be suggested including as yet unrecognized conjugates but in the absence of experimental evidence these are fruitless speculations. We have not attempted a detailed exploration of these excretion products because there seemed so little relation to the fate of the hormone in the human species.

It is apparent that in so far as testosterone is concerned the liver of the

rodent is one of the principal excretory organs. The administered hormone and/or its metabolites reached the gastrointestinal tract by way of the bile as evidenced by the high level of radioactivity in the gall bladder of mice. The majority of the radioactivity was found in progressively lower segments of the bowel as time after injection was prolonged and conversely the radioactivity in the liver and gall bladder diminished with time. There is some evidence of reabsorption of radioactivity from the intestinal tract as can be seen from the comparatively low level in urine 65 minutes after injection together with a rather large amount in the gut at this time. The urine and fecal radioactivity at longer intervals after administration shows lesser amounts in the bowel and larger amounts in the urine. These results are admittedly inconclusive for the existence of an enterohepatic circulation but they suggest that the hormone or the metabolites are to some extent reabsorbed.

Certain other observations on the fate of C^{14}-testosterone were made in the course of this survey. The tissue distribution and excretion of radioactivity in a male C_3H normal mouse was compared with a male mouse of the same strain bearing a transplanted mammary adenocarcinoma. The results are recorded in Table 6. From this comparison it is clear there was little difference between the normal and the tumor bearing animal and further that there was no marked localization of radioactivity in the tumor. The results are in good agreement with the general finding throughout the survey that there was only a minor amount of activity in any tissue even in the short interval of 65 minutes after injection.

One other experiment deserves brief mention, namely the comparison of a normal and castrate mouse with regard to the excretion of testosterone. It was found, in the one instance examined (Table 5), that there was a considerably greater excretion of radioactivity in the urine of the castrate. This preliminary result requires confirmation and elaboration.

Finally we should like to re-emphasize that the discussion and conclusions reported with these experiments are restricted to the compounds and the species studied and especially to the route used for administration of the hormones. It is recognized that the surge of hormone resulting from intraperitoneal injection does not simulate the normal release from the gonad. On the other hand the experiments were not designed as tracer studies of endogenously produced testosterone and though certain inferences have been drawn related to this question it is quite clear that much further work remains before these can be firmly established.

SUMMARY

Following the intraperitoneal injection of C^{14}-4-testosterone and C^{14}-21-progesterone in mice and rats, radioactivity was rapidly excreted in both

urine and feces. Practically all of the administered radioactivity was recovered. About 10 per cent of the injected radioactivity was found in the respiratory CO_2 from the animals receiving progesterone whereas no measurable radioactivity was found in the respiratory CO_2 after testosterone treatment. The tissue distribution of radioactivity in mice after testosterone administration was studied at various time intervals and no specific localization of radioactivity was found in any organ or tissue save those concerned with the excretion of the hormone or its metabolites. Most of the radioactivity was recovered from the feces or the contents of the gastrointestinal tract. The evidence indicated that the majority was excreted in the bile and that reabsorption from the gastrointestinal tract occurred. Chemical fractionation of the excreted radioactivity in both the urine and the feces showed that the greater portion was in the form of water soluble compound(s) which remained so even after vigorous acid or alkaline hydrolysis; only a minor portion of the excreted radioactivity could be obtained in an ether soluble form. The conclusion was drawn that the fate of these hormones in rodents does not afford information directly applicable to the problem of steroid hormone metabolism in humans.

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EFFECTS OF PROPYLTHIOURACIL ON THE GROWTH OF YOUNG MALE GUINEA PIGS[1]

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THYROPARATHYROIDECTOMY of the young rat has been found to result in a general dwarfing (Hammett, 1929; Salmon, 1936, 1938). Following thyroidectomy (Scow and Simpson, 1945), rats developed slowly; differentiation was possible in the absence of the thyroid, but growth proceeded at a slower rate, with retarded opening of the eyes, late eruption of the teeth, and delayed pubescence. Thiouracil or thiourea treatment of young rats (Hughes, 1944; Williams, Weinglass, Bissell, and Peters, 1944; Leathem, 1945, 1946; Barker, 1949) resulted in dwarfing or growth retardation. No significant effect was observed on the relative weights of the pituitary, gonads, kidneys, spleen, liver, or adrenals. Propylthiouracil treatment of young rats (Goddard, 1948) also resulted in growth retardation with no effect on general health, sexual maturation, hematology, or behavior. In all the cases cited above, where goitrogenic drugs were used to induce a hypothyroid state, the thyroid glands showed a large increase in weight.

In contrast to the effect on the rat, propylthiouracil administration affected young beagle puppies only by increasing the weight of the thyroid glands (Mayer, 1947). Body weight, general appearance and behavior, relative weights of the brain, heart, kidney, spleen, liver, parathyroids, pituitary, testes, and adrenals were unaffected.

The thyroids of male rabbits to which thiouracil was administered for 16 weeks beginning at 4 weeks of age do not seem to have been examined but the onset of sexual maturation was arrested (Maqsood, 1951). Degenerative changes were seen in the seminiferous tubules, the epithelial cells lining the epididymis were decreased in height, sexual desire was absent, and there was no ejaculation when an artificial vagina was used.

Very little in the way of comparable studies in the guinea pig have been reported. D'Angelo (1950) found that feeding high doses of propylthiouracil to females weighing about 250 grams at the beginning of a 60-

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to 78-day treatment period did not affect the body weight, although the increase in relative thyroid weights was on the order of 300%. The offspring of female guinea pigs treated with propylthiouracil during gestation exhibited tremendously enlarged thyroids (Albrieux, Estefan, and Gonzalez, 1946; Webster and Young, 1948; Webster, 1949 a). Peterson (1950) showed that propylthiouracil passed the placenta to affect the thyroid of the fetus directly. Since it is unlikely that neither the mothers nor their offspring were then able to produce thyroid hormone, the fetuses probably developed in the absence of the hormone. Despite this, their birthweights were normal as was their subsequent development (Webster, 1949 b). However, it cannot be said that the offspring of thiouracil-fed mothers are necessarily lacking in thyroid hormone after birth, since it has been shown that the regression of the goiters is followed by a reorganization of the gland (Webster and Young, 1948; Webster, 1949 b). The present study was undertaken in order to determine the effects of feeding propylthiouracil to male guinea pigs, beginning shortly after birth.

METHODS

Thirty mg. of 6-n-propyl-2-thiouracil[3] per kilogram of body weight were given daily by medicine dropper to 30 male guinea pigs (Louisville stock) from within 12 hours after birth until the animals were killed 4 to 16 weeks later. Since the drug was dissolved in dilute ammonia, the 19 controls received dilute ammonia solution which corresponded in concentration and volume to that received by the experimental animals. At no time was a toxicity to the ammonia manifested. The daily dose, given in two equal portions, one in the morning and the other in the evening, was adjusted daily and individually to body weight. It is estimated that not more than 0.05 ml. of a portion (0.5 to 1.0 ml.) was lost in force-feeding an animal.

The birthweights of the animals ranged between 70 and 103 gm.; the average birthweights of the experimental and control animals were 87 and 85 gm., respectively. Where possible, littermates were distributed between the two groups. The animals were kept under the standard conditions of the colony, the temperature being maintained between 70° and 75° F. The diet consisted of oats and Purina rabbit pellets *ad libitum* with cabbage and alfalfa hay once a day. Drinking water was available at all times. All animals were weaned at 25 days regardless of weight.

Three control and 6 experimental animals were killed when they were 4 weeks old and 4 controls and 6 experimentals at each of the following ages: 6, 8, 12, and 16 weeks.

In order to ascertain the time of sexual maturation as defined by the age at the first fertile mating (Webster and Young, 1951), the animals of the 16-week group were observed in mating tests (Young and Grunt, 1951) made at approximately 4-day intervals beginning the fiftieth day of age.

At the time of sacrifice, an animal was anesthetized with ether, the abdomen opened, and a heart puncture made through the diaphragm. As much blood as possible was removed with a 10 cc. syringe for serum protein-bound iodine (PBI) determinations. The thyroid, testes and epididymides, spleen, kidneys, adrenals, and heart of each animal was removed in that order, trimmed of fat and placed in a moist chamber to minimize

[3] Probacil supplied by Lederle Laboratories, courtesy of Dr. Stanton M. Hardy.

loss of weight due to water evaporation. They were weighed as rapidly as possible on a torsion balance. Those organs whose weights were greater than two grams, usually the kidneys and gonads, were weighed in weighing bottles on an analytical balance.

The method of determining serum PBI levels was slightly modified after that of Salter and Johnson (1948):

1. 2.8 N H_2SO_4 instead of 1.4 N.

2. 0.1 N arsenious acid was made up by dissolving 0.495 gm. of As_2O_3 in 2.7 ml. 4 N NaOH, diluting to almost 100 ml. with 0.03 N H_2SO_4. 0.86 ml. concentrated HCl was added and the total brought to volume (100 ml.).

3. Instead of using Klett-Summerson colorimeter tubes, 13 mm. $\times$100 mm. pyrex test tubes were calibrated for use in the colorimeter.

4. The tube-holder of the machine was adapted for the smaller tubes by placing a cork in the bottom of the well. The height of the cork was such that the curved part of the bottom of the colorimeter tube lay just below the light aperature and the surface of a 2.5 ml. volume of liquid lay just above.

5. In preparing an ashed sample for the colorimetric determination of iodide, 1.5 ml. instead of 3.0 ml. distilled water was added to the oxidation tube.

6. The blank consisted of 1.0 ml. distilled water, 0.5 ml. 4 N NaOH and 1.0 ml. 2.8 N H_2SO_4.

7. After the addition of 0.25 ml. arsenious acid to each tube, all tubes except the blank were centrifuged. A stop-watch was started at the time of addition of 0.125 ml. ceric ammonium sulfate to the first tube. Subsequent tubes received the same amount of the reagent at 15-second intervals, the time of addition being recorded.

8. In calculating the results the readings for a tube were plotted against the corresponding times on semi-logarithmic paper and the slope of the resulting straight line calculated from the formula,

$$\text{slope} = \frac{\log_e \dfrac{\text{high reading}}{\text{low reading}}}{\text{difference in time } (t_2 - t_1)}$$

The slope of the blank was subtracted from that of each sample before referring the latter to a nomogram for interpretation in terms of iodide concentration. The nomogram was obtained by determining the slopes of standard iodide solutions and plotting the results against the corresponding iodide concentration expressed in μg. per cent on double logarithmic paper.

RESULTS

Average values of the daily body weights of each group of animals were plotted against age (Fig. 1). The growth curve of the propylthiouracil-fed animals lies slightly but consistently below that of the controls, but the degree of growth retardation is so slight as to be of doubtful significance.

When relative organ weights are compared it is evident that the thyroid weights of the propylthiouracil-fed animals increased markedly with age, while those of the controls remained about the same or decreased somewhat (Table 1). The average relative weight was greater in the experimental group than in the control for the first time at the sixth week,

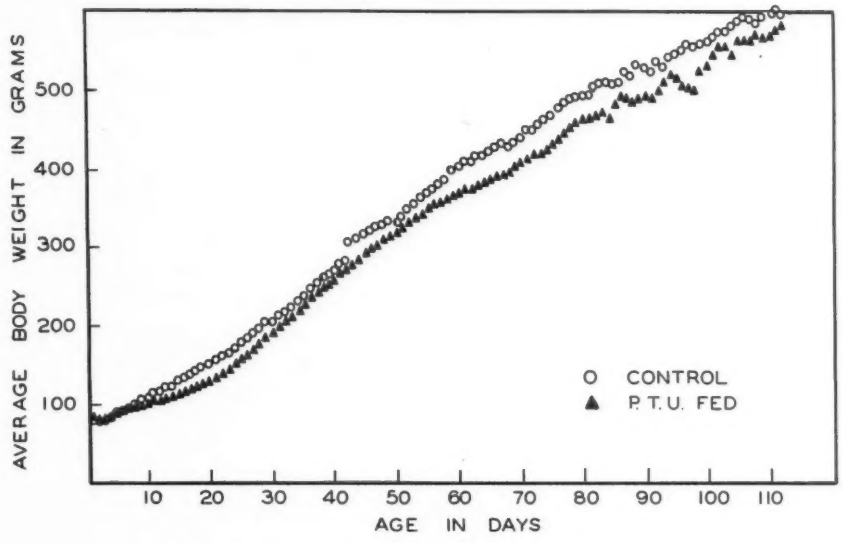

FIG. 1. Growth curve of male guinea pigs fed 30 mg./kg. propylthiouracil daily from birth.

although the difference was not statistically significant until the eighth week (Student's "t" test). However, the microscopic appearance of the thyroids in the 6-week old propylthiouracil-treated animals was typical of that resulting from goitrogen treatment; *i.e.*, epithelial hyperplasia and depleted colloid. The treatment had no specific effect on the relative weights of the testes and epididymides, spleen, kidneys, heart, or adrenals. Student's "t" test applied to the relative gonad and kidney weights at

TABLE 1. ORGAN WEIGHTS (MG./100 GM. BODY WEIGHT) OF YOUNG MALE GUINEA PIGS FED PROPYLTHIOURACIL

Age in weeks	Body weight		Thyroid		Testes and epididymides		Spleen	
	Control	Exp.	Control	Exp.	Control	Exp.	Control	Exp.
4	201±27.2	180± 9.0	17.4±1.8	16.3± 1.2	163±19.0	144±15.7	130±11.9	151±28.7
6	242±44.6	276±18.2	15.1±1.7	23.0± 2.8	210±49.8	221±26.8	129±12.4	146±21.8
8	365±16.8	358±20.1	12.0±0.8	51.8± 8.1*	275±24.9	268±32.5	118± 7.0	122±11.3
12	524±25.4	465±16.8	15.7±1.3	81.6±13.4*	543±51.9	543±12.9	134±22.8	111± 9.0
16	594±42.3	578± 7.5	12.4±1.9	135.3±13.0*	643±39.3	667±18.4	111± 6.6	115± 5.0

Age in weeks	Body weight		Kidneys		Adrenals		Heart	
	Control	Exp.	Control	Exp.	Control	Exp.	Control	Exp.
4	201±27.2	180± 9.0	788±173.7	890±33.7	43.3±4.5	45.3±5.2	352± 9.8	383±14.7
6	242±44.6	276±18.2	922±136.7	815±27.2	37.3±8.5	35.7±1.3	372±17.6	366± 9.6
8	365±16.8	358±20.1	733± 38.5	818±33.8	35.1±2.3	38.4±2.9	277±44.6	349±21.0
12	524±25.4	465±16.8	617± 26.2	676±28.4	41.1±2.7	41.1±2.4	301± 5.4	280±10.5
16	594±42.3	578± 7.5	596± 7.7	646±11.6	45.6±4.6	44.7±3.7	289± 7.7	293±10.4

The ± values are the standard error of the means.
* The difference from the control value is significant at the 1% level of confidence (Student's "*t*").

various ages indicated that the differences between mean values are not significant.

One animal from each of the control and experimental groups did not mate. Therefore, these two animals were not considered in computing the average age of sexual maturation. The average age at which the first fertile mating occurred in the three controls that mated was 89 days (79 to 106); the corresponding figure for the five propylthiouracil-fed males was 88 days (78 to 90). These data are in accordance with figures obtained by Webster and Young (1951). Propylthiouracil obviously had no effect on sexual maturation. The offspring resulting from these matings were normal in appearance and activity.

TABLE 2. SERUM PROTEIN-BOUND IODINE LEVELS IN YOUNG MALE GUINEA PIGS FED PROPYLTHIOURACIL

Age in weeks	Control serum PBI μg. %	Experimental serum PBI μg. %
4	3.0±0.10	2.9±0.29
6	2.6±0.79	2.6±0.54
8	2.0±0.72	2.2±0.49
12	2.1±0.31	2.2±0.26
16	2.7±0.37	2.8±0.26

The ± values are the standard error of the means.

Table 2 shows the mean PBI values obtained for each group at each age level. It is evident that propylthiouracil had no effect on PBI levels within the limits of the analytical procedure used.

DISCUSSION

The administration of antithyroid drugs to the rat resulted in a pronounced retardation of growth (Hughes, 1944; Williams, Weinglass, Bissell, and Peters, 1944; Goddard, 1948; Barker, 1949). This is a different effect from that produced in the guinea pig, in which growth retardation was slight, if any. However, in neither species were relative weights of the kidneys, spleen, gonads, or adrenals affected.

The difficulty in comparing one piece of work with any other is that too often the conditions under which the studies were performed were sufficiently different to account for many of the dissimilar results. However, the experimental conditions employed by Mayer (1947) and Goddard (1948) in their studies of the beagle puppy and rat make possible a comparison with the work on the guinea pig. In the three cases, propylthiouracil was used, the dose was 30 mg. per kilogram body weight per day, and the animals were young. Goddard began his treatment of rats on the eleventh day, Mayer treated his puppies from the thirty-third day, and treatment of the guinea pigs began the day of birth.

The ratios of the average relative thyroid weight of the experimental to the control equivalent at 112 days of age for the rat and guinea pig and a roughly corresponding age for the beagle puppy are 17.5, 10.9, and 3.5, respectively. It is evident that there was a considerably greater hypertrophy of the thyroid in the rat than in the other two species, the beagle puppy being the least affected. The ratios of average body weight in the experimental animals to that in the controls are 0.380 for the rat, 0.973 for the guinea pig, and 1 for the beagle. Again, the greatest effect is seen in the rat, while the beagle puppy and the guinea pig seem to have been unaffected or very nearly so. Thus, in the rat, thyroid hypertrophy and growth retardation were observed; in the beagle puppy there was only an hypertrophy of the thyroid. The picture in the guinea pig more nearly resembles that in the latter; the only effects were thyroid hypertrophy and a possible slight retardation of growth.

A comparison of this study to that of D'Angelo (1950) in the female guinea pig is of interest. Although the weight increase of the thyroids of both the females (250 gm.) and the males (newborn) was on the order of 300%, no difference was found in the body weight of the females and at the most only a possible slight decrease in the males.

The almost complete lack of reaction of the male guinea pig to propylthiouracil is rather striking. From both published (Brown, Peterson, Rayner, and Young, 1950) and unpublished work from this laboratory it would appear that the guinea pig maintained at an environmental temperature of 70° to 75° F. requires only a low level of thyroid activity. This is suggested by the failure of propylthiouracil to affect growth in the young male or gain in weight in the adult. The strength of sex drive of the adult male is not lowered as a result of thyroidectomy (Young, Peterson, Rayner, and Brown, 1950). In addition to oxygen consumption which was decreased 8%, heart rate and serum PBI of adult males after thyroidectomy were depressed (Peterson, Rayner, Brown and Young, 1952). Depression of oxygen consumption following thyroidectomy or goitrogen treatment, on the other hand, amounted to −25% to −40% in the rat (Meyer and Ransom, 1945) and −14% to −38% in the rabbit (Krichesky, 1935; Sachs, 1939). A similar explanation might account for the lack of difference between PBI levels of the propylthiouracil-treated males and their controls. The method of determination however is probably not sensitive enough to detect what must be a very slight depression in iodine values which are already at a low level.

SUMMARY

Thirty mg. propylthiouracil per kilogram per day were fed to male guinea pigs from the day of birth until sacrifice at 4, 6, 8, 12, or 16 weeks of age. The very slight retardation of growth was of doubtful significance.

The thyroids showed an increase in weight. No effect was found on the relative weights of the testes and epididymides, kidneys, adrenals, spleen, or heart, or on the age of sexual maturation. No effect was found on serum protein-bound iodine levels within the limitations of the method used. It is suggested as an explanation for the general lack of effect that the guinea pig maintained at an environmental temperature of 70° to 75° F. requires only a low level of thyroid activity and therefore is not dependent on thyroid hormone for many vital processes including growth.

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METABOLIC AND TOXICITY STUDIES ON PARA-HYDROXYPROPIOPHENONE

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INTEREST was recently aroused concerning the pharmacology of para-hydroxypropiophenone (PHP) by reports of Buu-Hoï and associates (1950). This well-known compound was believed to exert a number of actions on the endocrine system which if true might have therapeutic value. In fact, early clinical trials by Perrault *et al.* (1949) indicated that such was the case. Earlier work with PHP, which is a precursor of stilbestrol, had indicated that the former was essentially actionless except for a weak estrogenic activity (Emmens, 1941). The following report deals with toxicologic, metabolic, and fertility studies in experimental animals in an attempt to verify the findings of the French workers.

EXPERIMENTAL STUDIES

1. *Chemical properties*

The PHP employed was a purified preparation obtained from the Sumner Chemical Company Inc. It is a white crystalline compound relatively insoluble in water and slightly soluble in alcohol and ether. It melts at 153–55° C. We checked our material against some of the French PHP (Frenantol tablets) and could detect no difference chemically.

2. *Acute toxicity*

The median lethal dose by intravenous injection was determined in starved albino mice. In order to get sufficient PHP in solution, sodium hydroxide was added. The $LD_{50} \pm$ S.E. on two separate tests was 160 ± 6.8 and 153 ± 5.8 mg. of PHP per kg. of body weight. Death occurred immediately following injection, being preceded by generalized convulsions. The animals were kept for 12 days after injection; no delayed deaths resulting.

3. *Chronic feeding experiments*

Young female rats about a month old were given our regular stock diet to which PHP was added. A total of 40 animals, divided into 4 groups of 10 rats each, was used. The concentration of PHP in the food of 3 groups was 1.0, 0.5 and 0.1%. The fourth group was a control receiving only the

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regular diet. Blood studies consisting of erythrocyte, leucocyte and differential counts and hemoglobin determinations were made on three animals from each group at the start and again after one month on the diet. At regular intervals, body weights were recorded to measure growth rate.

As shown in Table 1, there was no difference in the rate of growth of control and PHP-fed rats for periods up to 5 months in either first or second generation animals. No change was found in erythrocyte and hemoglobin values on any level of PHP in the diet. A moderate leukocytosis

TABLE 1. EFFECT OF PHP IN DIET ON GROWTH AND FERTILITY OF MALE AND FEMALE RATS

	Group	Average weight (gm.)			Mating record		Litter Data			
		Start	1 month	4 months	No. mated	No. pregnant	Av. No. in litter	Av. No. lived	Average No. surviving per litter	
									Males	Females
First Generation—♀	Control	64	119	183	5	3	8.3	4.3	3.0	1.3
	1.0% PHP	61	122	176	4	4	8.5	8.0	4.8	3.3
					2*	1	9	6	2	4
	0.5% PHP	54	113	200	5	5†	7.8	3.8	2.3	1.5
					2*	1	10	7	5	2
	0.1% PHP	50	123	195	5	4	9.0	6.3	4.3	4
		6 weeks		5 months						
Second Generation		Males	Females	Males	Females					
	Control	68	62	255	187	4‡	2	4.5	0	
	1% PHP	61	67	244	172	5‡	4	7	3	3.0	3.5
	0.5% PHP	57	64	241	174	5‡	3	6.3	3.3	3.0	2.0
	0.1% PHP	78	78	266	181	Not Mated					
Third Generation	Control	None survived								
	1% PHP	Growing normally—Average weight not calculated because of age variance								
	0.5% PHP	Growing normally—Average weight not calculated because of age variance								

* Bred to male rats on 1% PHP for similar period.
† 1 rat died during pregnancy.
‡ Intermated—both males and females on same diet.

due to increased lymphocytes occurred in all groups including controls and was considered as unrelated to the drug.

At the end of the first month two rats on the 1% PHP diet were killed for autopsy. At the same time, a control animal was also sacrificed. Subsequently, post-mortem examinations on two more rats were made at intervals of $2\frac{1}{2}$ and $3\frac{1}{2}$ months from the onset. These rats also had received the highest dose of PHP. Gross and microscopic examinations, including thyroid glands and ovaries, revealed the tissues of all these animals to be entirely normal.

At the conclusion of the experimental study, all surviving animals were killed. This included the third generation rats, which were from 2 to 6 weeks old. Gross autopsies on these animals were normal.

4. *Antigoitrogenic action*

Adult male rats were given thiouracil or thiouracil plus PHP in their diet or water for periods of 14 to 28 days. At the end of the period, the animals were sacrificed and the thyroid glands carefully removed and weighed. A control group on our regular diet was similarly studied. Results are shown in Table 2. PHP did not inhibit the goitrogenic effect of thiouracil.

TABLE 2. EFFECT OF PHP ON GOITROGENIC ACTION OF THIOURACIL IN RATS

Group	No. of animals	Regimen	Duration (days)	Average body weight (gm.)		Average thyroid weight (mg.)		
				On starting diet	At autopsy	Wet	Dry	Per 100 gm. body weight
I	4	0.1% Thiouracil in water 1.0% PHP in diet	14	300	299	34.0	7.50	11.40
II	5	0.1% Thiouracil in diet 0.1% PHP in diet	14	272	270	40.0	8.24	14.74
III	5	0.1% Thiouracil in water	14	273	281	26.5	6.22	9.44
IV	5	Normal diet	14	270	295	14.2	4.20	4.84
V	5	0.1% Thiouracil in water and diet	11	282	283	30.6		10.8
VI	5	0.1% Thiouracil in water 0.1% Thiouracil in water and diet	16 11	267	263	46.9		17.7
VII	5	0.1% Thiouracil in water and 1.0% PHP in diet 0.1% Thiouracil in water and diet and 0.1% PHP in diet	17 11	283	268	46.1		17.3
VIII	5	0.1% Thiouracil in water and 0.1% PHP in diet 0.1% Thiouracil in water and diet and 1.0% PHP in diet	17 11	291	277	39.4		14.2

5. *Effect on basal metabolic rate*

Four adult male rats received PHP in the diet for a period of 49 days to determine its effect on basal metabolic rate. Previously, the B.M.R. was measured for 3 consecutive days to ascertain the control level. Metabolic rate was determined in a Benedict-type, closed-circuit apparatus (Benedict, 1930) as modified in our laboratories for thyroid assays. For the first 2 days, two rats were given 600 mg. of PHP per kg., while the other two animals received 60 mg. of PHP per kg. These doses were given by stomach tube. Thereafter, the animals were fed diets with 1% and 0.1% PHP respectively. Figure 1 shows the individual results. At the start, there was a slight but definite increase in B.M.R. in three of the animals. Subsequently, a slight fall occurred in the 53-day period. This fall was not significant as it was similar to that seen in normal rats over the same period (Kroc and Stasilli).

6. *Studies on fertility and pregnancy*

Female rats of the first generation chronic feeding experiments were mated with normal males to determine the effect of PHP on fertility and

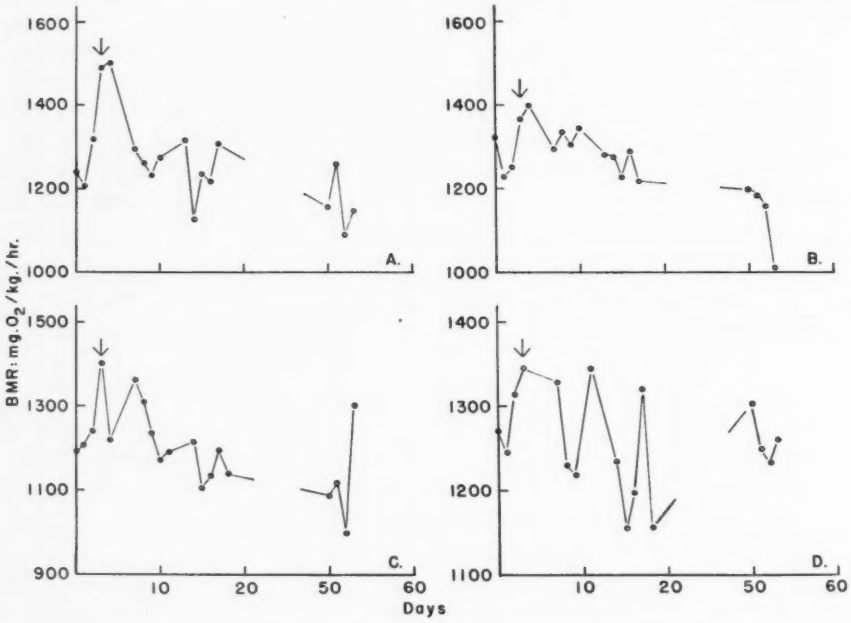

FIG. 1. Effect of PHP on basal metabolic rate of mature male rats. Administration of PHP was started at the arrow and continued for the remainder of the study. Rats A and B received a diet containing 1% PHP, while rats C and D were given 0.1% PHP.

pregnancy. In addition, some of these first generation females were mated with males which received 1% PHP in their diet for a similar period. The offspring of the first generation were subsequently intermated, so that all the animals except controls had received PHP for their entire lifetimes. The rate of pregnancy, size of litters, and number of surviving males and females are given in Table 1. This shows that into the third generation, PHP does not suppress fertility or inhibit pregnancy.

DISCUSSION

These studies do not lend support to the findings of the French investigators that PHP inhibits either gonadotropic or thyrotropic hormones from the pituitary. Our results are essentially in agreement with those of Money and associates (1951). However, they did find a decrease in uptake of I^{131} by the thyroid glands of animals treated with PHP. Their dosage was approximately the same as reported here, but our experiments were of longer duration. Schaffenburg, Masson, McCullagh and Haour (1951) found a decreased thyroid weight in one group of their animals which re-

ceived PHP. They attributed this to non-specific toxicity of the drug. Since our rats were older and showed no significant weight change, their interpretation would tend to be substantiated.

Assay of the estrogenic and gonadotropic-inhibiting potencies of PHP has recently been reported by Hertz and Tullner (1951). They found PHP to have 0.1% of the estrogenic activity of estrone and less than 0.5% of the gonadotropic-inhibiting effect of this estrogen. From their study they concluded that PHP does not have pituitary-depressant properties out of proportion to its estrogenic action.

PHP may be considered to be a relatively non-toxic compound. No attempt was made to measure the dose of PHP accurately on a weight basis in the chronic feeding experiments. From experience, however, it is possible to calculate that rats receiving one per cent of PHP in their diet are getting a dose of 500 to 1000 mg. per kg. daily. This is much larger than therapeutic dosages in human beings and was judged great enough to show results in experimental animals.

CONCLUSIONS

Para-hydroxypropiophenone shows no effect on growth, blood picture, metabolic rate, or fertility of rats receiving large doses in their food over periods up to 5 months. These findings do not support the concept that para-hydroxypropiophenone inhibits pituitary gonadotropin and thyrotropin.

Acknowledgments

Grateful acknowledgment is given to Mr. Kenneth Back, Mrs. Frances Mackenzie and Mrs. Jean Mattson for technical assistance.

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THE EFFECTS OF VARIOUS DOSES OF RADIOACTIVE IODINE ON THE FUNCTION AND STRUCTURE OF THE THYROID OF THE RAT

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THE purpose of this investigation was to study simultaneously the anatomical and the functional changes in the thyroid gland of growing rats following the administration of graded doses of radioactive iodine. The various aspects of the study embrace the following:

1. The initial uptake of a variety of doses of radioactive iodine by the thyroid, the rate of loss of the labeled activity from the thyroid, and the determination of the amount of radiation delivered to the thyroid.

2. The subsequent functional capacity of the irradiated thyroid gland as measured by its response to: a) A low iodine diet, b) Thiouracil, and c) Thyroid stimulating hormone (TSH) administration.

3. Growth of the animals as judged by the gain in weight and length of the body.

4. Histological changes associated with the above observations: damage, recovery, regeneration and possible tumor formation during prolonged periods of observation.

Among the reports concerning the damage to the thyroid produced by radioactive iodine (Hamilton, 1942; Gorbman, 1947; Findlay and LeBlond, 1948; Gorbman, 1949, 1950; Goldberg *et al.*, 1950; Winchester *et al.*, 1949), varying degrees of attention have been paid to factors which have a bearing on the changes produced. These factors include the age of the animal, the amount of dietary and "carrier" iodine, the functional and morphological state of the thyroid at the time of radiation, the dose of radioactive iodine administered and the amount retained per unit weight of thyroid tissue, and the total amount of radiation (roentgen equivalents physical) received.

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Gorbman (1950) has shown that 200 microcuries of radioactive iodine destroyed the thyroid of mice receiving a diet containing moderate amounts of iodide, whereas 28 microcuries destroyed the thyroid of such animals on a low iodide diet. The maximal labeled iodine uptake in the thyroid of animals on the former diet was 6–7.5 per per cent and on the latter diet was 58 per cent. In each instance the thyroid was destroyed by 4 microcuries of I^{131} per milligram of thyroid tissue.

In spite of the frequent use of tracer doses of radioactive iodine in animal experimentation, there is still uncertainty concerning the maximal dose of this isotope which may be given without producing functional or anatomical damage to the thyroid. Skanse (1948) observed in our laboratory that the normal growth of the chick thyroid was impaired by as little as ten microcuries of radioactive iodine (13,000 rep delivered); that the ability of the thyroid to concentrate iodine from food and water was affected by doses of 50 microcuries of radioactive iodine (60,000 rep delivered); that growth of the thyroid in response to thiouracil was impaired by 10 microcuries and completely inhibited by 50 microcuries; and that thyroids of animals given 10 microcuries did not increase in weight but did show a normal loss of iodine when thyroid stimulating hormone was administered. The latter two abnormalities were more pronounced at longer intervals of time following the radiation. Feller, Chaikoff, Taurog and Jones (1949) observed no functional change in the iodine metabolism of rats 10 days after the administration of 30 microcuries of radioactive iodine (28,000 rep delivered).

Hamilton in 1942 noted the presence of fibrosis and the lack of epithelial regeneration in the thyroids of dogs 100 days after the administration of 300 microcuries of radioactive iodine (I^{130}) per kilogram of body weight. The parathyroid and all other tissues were reported to be undamaged. It was estimated that 15,000–25,000 rep were delivered to the thyroid. Gorbman in 1947 administered doses of 100 to 1000 microcuries of radioactive iodine (I^{131}) to inbred mice that had been placed on a Purina Fox Chow diet. He observed that although the largest dose of radioactivity was almost completely lethal to the thyroid in a few days after its administration, the smallest dose destroyed 90 per cent of the gland in 120 days. He described injury to the parathyroid, the tracheal epithelium and to the recurrent laryngeal nerves. Findlay and LeBlond (1948) placed two female rats on a low iodine diet for 10 weeks and then administered doses of 78.5 and 60.8 microcuries of radioactive iodine (I^{131})—20,000 rep delivered. Histological study of the thyroids revealed transformation of central follicles into solid cell groups with loss of colloid, cloudy swelling of the cells with variation in size of the nuclei, increased interstitial fibrous tissue accompanied by lymphocytic infiltration, edema, fibrillation of the colloid,

and changes in the tracheal epithelium and in the parathyroid. Goldberg *et al.* (1950) have described a sequential series of histological changes induced in the normal thyroid of the rat. No evidence of damage to the thyroid was observed at any interval after the injection of 18 microcuries of radioactive iodine (12,000 rep[1] delivered). Progressive stages of degeneration were described in the thyroids of animals receiving 300 (110,000 rep[1]), 525 and 875 (330,000 rep[1]) microcuries. Histological changes were noted as early as 12 hours after the administration of 525 or 875 microcuries of this isotope and as early as 24 hours after 300 microcuries. Cells similar to the so-called Hurthle cells were noted 5 to 8 months after the injection of 525 microcuries of radioactive iodine. The parathyroid glands were found to be quite resistant to even the largest dose of radiation. Evidence of changes in the tracheal epithelium were noted.

The possibility of neoplastic change taking place in the thyroid glands of patients treated with radioactive iodine has been raised (Pemberton, Haines and Keating 1949; Trunnell, 1949; Werner, Quimby and Schmidt 1949). Although Doniach (1950) observed the formation of adenomas in the thyroid of control rats, he reported that there was an increased incidence of adenomas in rats one year after the administration of 32 microcuries of radioactive iodine (I^{131}) of which 33 per cent was retained in the thyroid. This incidence of adenoma formation was further increased by the simultaneous administration of goitrogenic drugs. It should be recalled however, that Griesbach *et al.* (1945) and Money and Rawson (1947) produced adenomas in the thyroids of rats with the administration of goitrogenic substances alone. Goldberg and Chaikoff (1951) observed the formation of adenomas in the thyroids of two out of ten rats 18 months after the administration of 400 microcuries of radioactive iodine. In the same publication, they reported that Moon observed six small adenomas in the thyroids of 14 control rats of the same strain. Although Goldberg and Chaikoff reported that the adenomas did not appear malignant, they entertained this possibility.

Chapman *et al.*, (1949, 1948), Williams *et al.* (1949), and Shapiro (1950) have briefly described histological changes which have taken place in the thyroids of patients following treatment with radioactive iodine for hyperthyroidism. Cellular swelling, falling in of the follicular cells, fibrosis and regenerative hyperplasia have been described. Nuclear changes are evident in some of the published photographs. Dobyns *et al.* (in press) have attempted to correlate function with histology and have noted no evidence of malignancy in thyroid tissue obtained as long as 8 years after the administration of the isotope.

[1] Delivered to a point at the center of the thyroid gland.

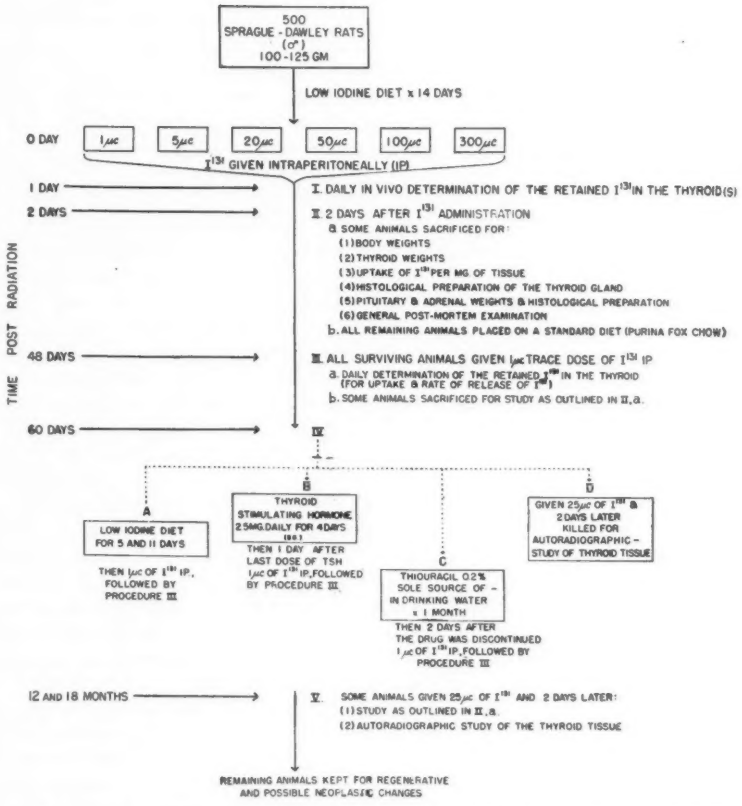
500
SPRAGUE - DAWLEY RATS
(♂)
100 - 125 GM
LOW IODINE DIET x 14 DAYS
0 DAY
1μc
5μc
20μc
50μc
100μc
300μc
I131 GIVEN INTRAPERITONEALLY (IP)
1 DAY
I. DAILY IN VIVO DETERMINATION OF THE RETAINED I131 IN THE THYROID(S)
2 DAYS
II. 2 DAYS AFTER I131 ADMINISTRATION
a. SOME ANIMALS SACRIFICED FOR:
(1) BODY WEIGHTS
(2) THYROID WEIGHTS
(3) UPTAKE OF I131 PER MG OF TISSUE
(4) HISTOLOGICAL PREPARATION OF THE THYROID GLAND
(5) PITUITARY & ADRENAL WEIGHTS & HISTOLOGICAL PREPARATION
(6) GENERAL POST-MORTEM EXAMINATION
b. ALL REMAINING ANIMALS PLACED ON A STANDARD DIET (PURINA FOX CHOW)
TIME POST RADIATION
48 DAYS
III. ALL SURVIVING ANIMALS GIVEN 1μc TRACE DOSE OF I131 IP
a. DAILY DETERMINATION OF THE RETAINED I131 IN THE THYROID (FOR UPTAKE & RATE OF RELEASE OF I131)
b. SOME ANIMALS SACRIFICED FOR STUDY AS OUTLINED IN II, a.
60 DAYS
IV
A
LOW IODINE DIET FOR 5 AND 11 DAYS
THEN 1μc OF I131 IP, FOLLOWED BY PROCEDURE III
B
THYROID STIMULATING HORMONE 2.5MG. DAILY FOR 4 DAYS (IM)
THEN 1 DAY AFTER LAST DOSE OF TSH 1μc OF I131 IP, FOLLOWED BY PROCEDURE III
C
THIOURACIL 0.2% SOLE SOURCE OF - IN DRINKING WATER x 1 MONTH
THEN 2 DAYS AFTER THE DRUG WAS DISCONTINUED 1μc OF I131 IP, FOLLOWED BY PROCEDURE III
D
GIVEN 25μc OF I131 & 2 DAYS LATER KILLED FOR AUTORADIOGRAPHIC - STUDY OF THYROID TISSUE
12 AND 18 MONTHS
V. SOME ANIMALS GIVEN 25μc OF I131 AND 2 DAYS LATER:
(1) STUDY AS OUTLINED IN II, a.
(2) AUTORADIOGRAPHIC STUDY OF THE THYROID TISSUE
REMAINING ANIMALS KEPT FOR REGENERATIVE AND POSSIBLE NEOPLASTIC CHANGES

FIG. 1. Schematic representation of the course of investigation in 500 rats following the administration of various doses of radioactive iodine.

MATERIALS AND METHODS

A total of 500 male rats (Sprague-Dawley), each weighing between 100 and 125 grams, were given a modified Remington diet[2] (low iodine) for 14 days. Distilled water and the diet were permitted ad libitum. The animals were placed in metal cages with screened flooring to prevent access to their excreta. Initially the animals were weighed and measured and individually marked for identification. The weight of the animals was determined at intervals throughout the experiment.

The various phases of the experimental procedures carried out on these animals are illustrated in Fig. 1. After preliminary preparation on the low iodine diet, the 500 rats

[2] This diet consisted of 50 grams of NaCl, 1000 grams Brewer's yeast, 150 grams calcium carbonate, 3800 grams corn meal and 1000 grams wheat gluten flour. This diet contained 0.090 micrograms of iodide per gram of food.

were divided into 6 groups; and each animal was then given a dose of radioactive iodine[3] by the intraperitoneal route according to the following schedule: Group I, 1 microcurie; Group II, 5 microcuries; Group III, 20 microcuries; Group IV, 50 microcuries; Group V, 100 microcuries; Group VI, 300 microcuries. Each dose contained 0.005 gamma of sodium iodide as carrier.

One day following the administration of the isotope, 12 animals were chosen at random as representatives from each group. Each day these animals were anesthetized by the intraperitoneal administration of pentobarbital sodium (3 milligrams per 100 grams of body weight), and the thyroid region was counted under reproducible geometrical conditions within a lead chamber which contained a shielded platinum screen Geiger-Mueller counter. With this geometry and this counter, 1 microcurie of radioactive iodine (standard) yielded an average of 1800 net counts per minute. The per cent uptake of radioactive iodine in the thyroid region was determined by measuring a standard identical to the administered dose of radioactivity and under the same geometrical conditions. The disappearance of the labeled iodine from the thyroid area was plotted on semi-logarithmic paper. From the exponential portion of this curve, the biological half-period was determined for each group of animals.

Two days after the administration of the initial dose of radioactive iodine, 5 animals were sacrificed from each group for studies as outlined in Fig. 1 (part II). The thyroid glands were removed and weighed on a Roller-Smith balance to one hundredth of a milligram. One lobe of the thyroid was then dissolved in 3 cc. of 5 per cent potassium hydroxide and the radioactivity of an aliquot of the digest was determined and related to a standard of the original dose given to the animal. The other lobe of the thyroid, which served for histological preparation, was fixed in Bouin's solution. Tissue sections were cut to 6 microns. At the time the animals were sacrificed, the pituitaries and adrenals were removed, weighed and prepared for histological examination. In addition, a general post-mortem examination was made on each animal.

Two days after the initial dose of radioactive iodine had been given, the low iodine diet was discontinued and all the animals were given the Purina Fox Chow diet[4] which was then continued throughout the entire experiment except for a brief period in one group of animals as outlined in Fig. 1 (Part IV A). There was a considerable difference in the amount of food consumed daily by relatively normal animals as compared with those that were made thyroid deficient. The former consumed approximately 10 grams and the latter consumed approximately 4 grams per day.

Forty-eight days after the initial dose of radioactive iodine had been given, a tracer dose[5] (1 microcurie) of the isotope was administered to determine changes in thyroid function (See Fig. 1, Part III).

Sixty days after the initial dose of radioactive iodine had been administered, groups of animals were divided into sub-groups for observations as listed in Fig. 1 (Part IV, A, B, C).

Observations have extended over a period of a year and a half in order to observe regenerative or possible neoplastic changes which might take place in these thyroids.

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[3] Standardization of the radioactive iodine was carried out at the Radioactivity center of the Massachusetts Institute of Technology by comparison with a Radium standard. The primary standardization was performed by coincidence counting.

[4] This diet contained 3.2 micrograms of iodide per gram of food.

[5] 0.005 micrograms of sodium iodide was given as carrier with this and all subsequent tracer doses.

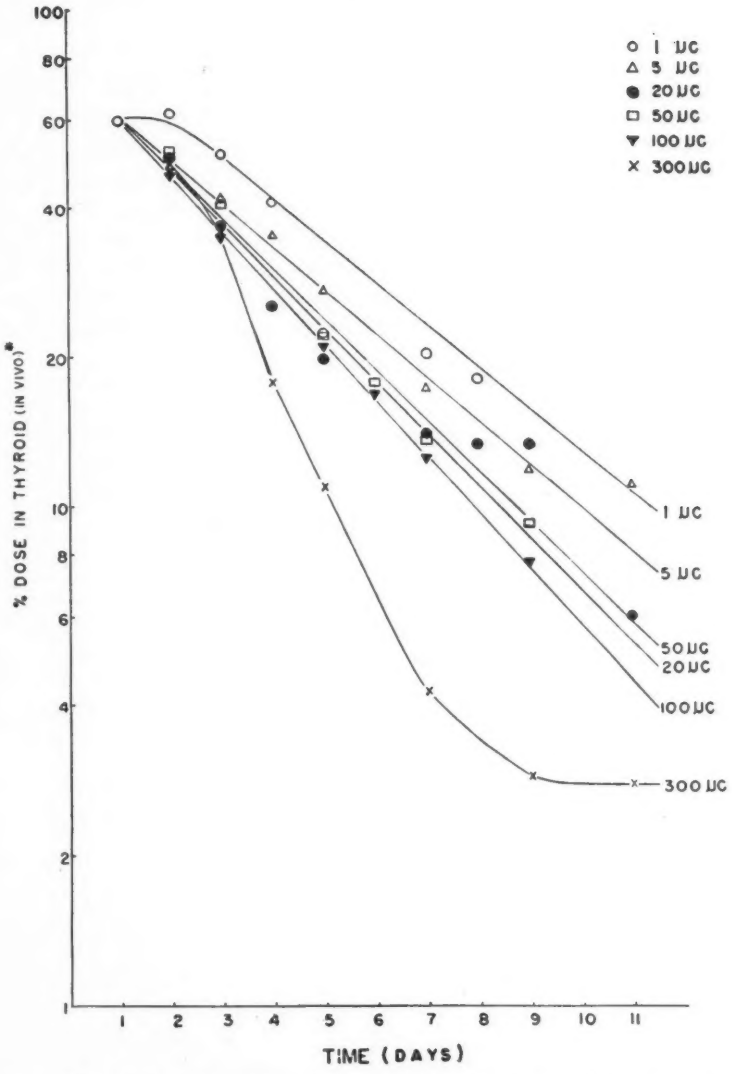

FIG. 2. Daily in vivo measurements of the retained radioactive iodine in the thyroid area were made over a period of 11 days following the administration of various doses of the isotope.

Each value represents an average percentage of 12 animals.

* This percentage is slightly modified in view of the normalization at 24 hours. (See text.)

RESULTS

Functional Studies

Experimental animals receiving the Purina Fox Chow diet retained about 6–8 per cent of a tracer dose (1 microcurie) of radioactive iodine in the thyroid region in 48 hours. After receiving the low iodine diet for two weeks, the uptake of iodine was substantially increased and relatively uniform among all the groups of animals (range 47–62 per cent).

Following the initial radiation dose of radioactive iodine, daily in vivo measurements of the amount of labeled iodine retained in the thyroid region were made over a period of 11 days. In Fig. 2, the uptake has been normalized to 60 per cent at 24 hours in order to demonstrate more clearly

TABLE 1. ESTIMATION OF THE TOTAL RADIATION (REP) DELIVERED TO THE THYROID GLAND OF RATS RECEIVING VARIOUS DOSES OF RADIOACTIVE IODINE

Dose (microcuries)	Biological half-period (TB)* (days)	Observed half-period (To)† (days)	Maximum total radiation dose delivered to the thyroid (rep)
1 μc.	4.4	2.8	1,800
5 μc.	4.0	2.7	5,800
20 μc.	2.9	2.1	30,000
50 μc.	2.9	2.2	80,000
100 μc.	2.7	2.0	197,000
300 μc.	1.4	1.2	288,000‡

* Biological half-period is the time necessary for one half of the stable iodine, which is labeled with the radioactive iodine, to leave the thyroid gland.

† Observed half-period was determined from the following equation: $1/To = 1/8 + 1/TB$.

‡ Average daily radiation dose delivered to the thyroid gland was totalled to determine this value. See text.

the difference in the rate of release. In general, the rate of loss of the labeled iodine from the thyroid gland increased with the amount of radioactivity administered. Animals that had received 300 microcuries of radioactive iodine lost the isotope from their thyroids much more rapidly after the third day than the animals that had received smaller doses of radioactive iodine. The very rapid loss of radioactive iodine from the thyroid of animals that received 300 microcuries of the isotope was in proportion to the histological changes that occurred in the thyroid. These will be described later.

From the exponential portion of these curves, the biological half-period was determined for each group of animals. With increasing doses of administered radioactivity, there tended to be a smaller biological half-period (See Table 1). The biological half-period of the animals that had received 300 microcuries is significantly different from all the others.

The average dose of radiation delivered on each successive day was determined by means of the following formula (Evans, 1947):

(1) $R = 62\ C\ \bar{E}$

R = roentgen equivalents physical per day (rep/day)
C = microcuries per gram of thyroid tissue
$\bar{E}$ = average energy of the beta ray = 0.205 mev.

The average daily dose of radiation was added to determine the total dosage. This formula was applied to data acquired from animals that had received 300 microcuries of radioactive iodine. When the decrease in radioactivity with time was approximately exponential, as in all other groups of animals, the total dose of radiation delivered to the thyroid gland was obtained by a simple mathematical integration of the above formula which yielded the following equation:[6]

(2) $D = 18\ CoTo$

D = total dose in roentgen equivalents physical (rep)
Co = initial concentration in microcuries per gram of thyroid tissue
To = observed half-period associated with the exponential decrease in activity (resulting both from decay and release).

The maximum total dose calculated from the formulas 1 and 2 is probably too large for the cells in the isthmus and in the periphery. The dose of radiation delivered to these cells has been calculated to be 0.42 per cent of the maximum dose (Feller *et al.*, 1949). Since the completion of this study, Dudley (in press) has devised a much more accurate method for determining the radiation dose to tissue. This is based on a quantitative radio-autographic analysis of the irradiated tissue. The maximum total dose of radiation delivered to the thyroid by the various doses of radioactive iodine as estimated by the above formulas is shown in Table 1. As will be subsequently demonstrated, we have observed functional changes in the thyroid with as small a dose as 5 microcuries which delivered 5,800 rep to the thyroid.

The response of the irradiated thyroid to a subsequent tracer dose of radioactive iodine: Forty-eight days after groups of animals had received doses of radioactive iodine ranging from one to 300 microcuries, they were given tracer doses (1 microcurie) of the isotope while on the Purina Fox Chow diet.

The forty-eight-hour uptake of radioactive iodine in the thyroid region in all groups of animals was approximately the same (5 to 8 per cent).[7] This range of uptake of radioactive iodine is interesting in view of the histologi-

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[6] We are indebted to Dr. Gordon Brownell for his aid in the mathematical analysis of these data.

[7] This uniform uptake of radioactive iodine in all groups of animals probably reflects the large amount of iodide in the diet.

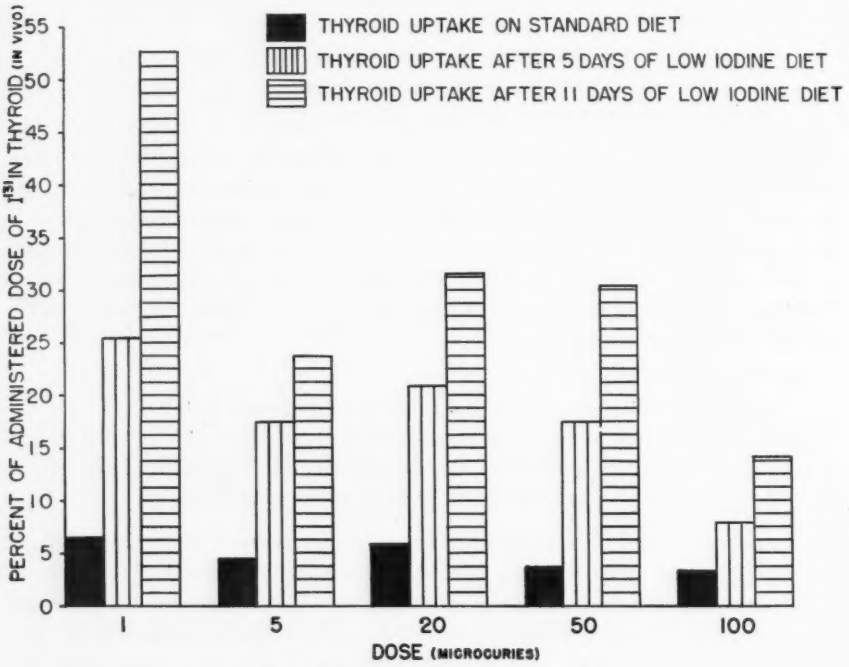


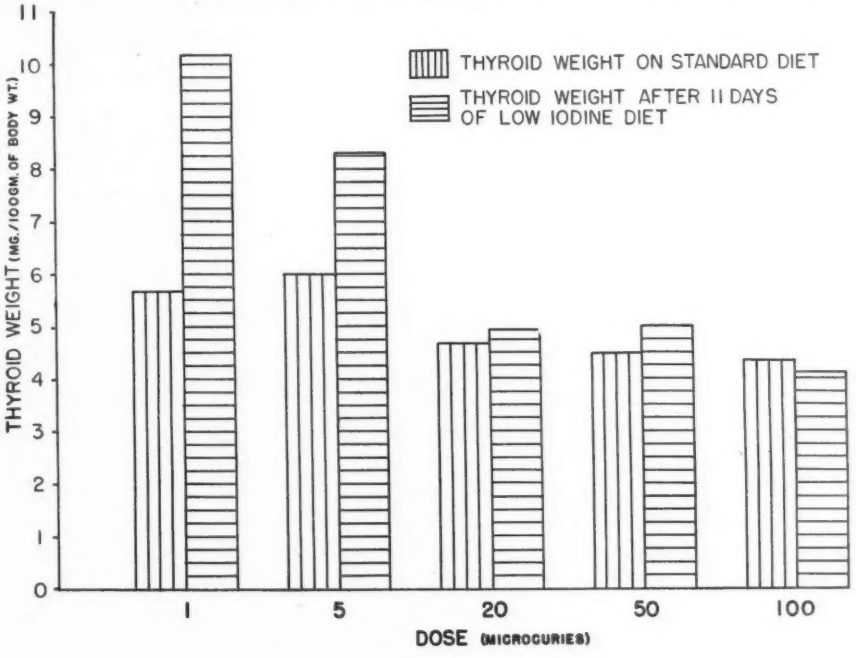
Fig. 3. The response of the irradiated thyroid gland of the rat to a low iodine diet (for 5 and 11 days) as measured by the 48-hour uptake of radioactive iodine in the thyroid area (in vivo).

cal picture of stimulation seen in these thyroids at this interval following radiation. (See Figs. 9 A & B).

The response of the irradiated thyroid to a low iodine diet: Sixty days after groups of animals had received doses of radioactive iodine ranging from one microcurie to 100 microcuries, the tracer dose (1 microcurie) procedure was repeated in some animals while still on the Purina Fox Chow diet. The 48-hour uptake of radioactive iodine in the thyroid region in all groups was 4 to 6 per cent.[7] Five animals from each group were then placed on the low iodine diet for 5 and 11 days, and the tracer dose procedure was repeated for each time interval (See Fig. 1, Part IV A).

Five days after the Purina Fox Chow diet had been replaced by the low iodine diet, there was a significant increase in the 48-hour uptake of radioactive iodine in the thyroid region in all groups of animals, but this increase was much less striking in animals which had received 100 microcuries of the isotope (See Fig. 3).

Eleven days after receiving the low iodine diet, the 48-hour uptake of radioactive iodine in the thyroid area of animals that had received one microcurie had reached 53 per cent, but the animals which had received 5, 20 or 50 microcuries took up significantly less. The most striking failure of the increase was noted in the animals which had received 100 microcuries (14 per cent uptake). Doses as small as 5 microcuries interfered with the subsequent ability of the thyroid to respond to a low iodine diet.



Sixty Days After I^{131} Administration

FIG. 4. The response of the irradiated thyroid gland of the rat to a low iodine diet (for 11 days) as measured by the change in thyroid weight.

After the animals had been on the low iodine diet for 13 days and the 48-hour uptake in the thyroid had been determined, the animals were killed by means of ether, and the thyroids were removed and weighed. It will be seen from Fig. 4 that there was a slight impairment in the ability of the thyroid to increase in weight in animals which had received only 5 microcuries. The thyroids of animals that had received 20, 50, and 100 microcuries of radioactive iodine demonstrated no ability to enlarge in response to this stimulus. Here again 5 microcuries of the isotope interfered with thyroid function.

The response of the irradiated thyroid gland to the administration of thyroid stimulating hormone (TSH): Sixty days after the animals had received the initial dose of radioactive iodine, five animals in each group were given 2.5 milligrams of TSH[8] subcutaneously each day for 4 days. Studies, as listed in Fig. 1 (Part IV B), were then carried out.

Analysis of the data on the thyroidal uptake of radioactive iodine, and on changes in thyroid weights revealed that there were no significant differences in any of these groups of animals. It is our opinion that the iodide in the Purina Fox Chow diet nullified any possible effects from the

TABLE 2. THE EFFECT OF TSH AND VARYING AMOUNTS OF IODINE IN THE DIET ON THE WEIGHT OF THE THYROID OF THE RAT

	Thyroid weights (mg/100 gm. of body wt.)
Low iodine diet×6 days	9.91±1.20*
Low iodine diet×4 days	8.32±1.12
Low iodine diet×2 days	8.75±0.55
Normal diet	7.81±0.36
TSH×4 days and normal diet	8.23±0.77
TSH×4 days and low iodine diet×1 day	11.47±1.22
TSH×4 days and low iodine diet×2 days	24.04±2.48
TSH×4 days and low iodine diet×4 days	20.38±2.68

* Each figure is the average weight of three thyroids and the standard error of the mean

TSH. That this lot of TSH was potent was proved by experiments in which the dietary intake of iodine was varied while TSH was being administered. While on the Purina Fox Chow diet there was no evidence of stimulation in the thyroids of normal control animals that were given the same amount of TSH. However, if the low iodide diet (0.090 micrograms/gram of food) was given on the last two of the four days that the TSH was injected, the weight of the thyroid of a normal animal increased threefold (See Table 2). Simultaneous histological studies revealed little to no evidence of stimulation in the thyroids until the dietary iodine was decreased.

The response of the irradiated thyroid to the administration of thiouracil: Sixty-four days after the animals had received various doses of radioactive iodine, five animals from each group were given a 0.2 per cent solution of thiouracil as their drinking water for the following thirty days. The thiouracil was then discontinued for 2 days and a tracer dose (1 microcurie) of radioactive iodine was given to each animal. Observations, as outlined in Fig. 1 (part IV C), were then carried out.

[8] The TSH preparation was made available through the courtesy of Dr. John R. Mote, of Armour and Co., Chicago, Ill. Each vial contained 25 mg. of (lot H71608) purified TSH of 4.5 Evans units per milligram. Adrenocorticotropin was present on a weight basis of 10 per cent of La-I-A. Gonadotropins, growth hormone, oxytocic and pressor substances, and prolactin were very low. The lyophilized TSH preparations were dissolved in 5 cc. of sterile saline and 0.5 cc. was administered daily to each animal.

The forty-eight-hour uptake of radioactive iodine in the thyroid area was 7 to 10 per cent in all groups of animals. Though the iodide in the Purina Fox Chow diet apparently interfered with the thyroidal uptake of radioactive iodine, it did not interfere with the ability of the thyroid to enlarge in response to the administration of thiouracil.

In Fig. 5 it will be seen that the thyroid weights of the animals, at the time that the original dose of radioactive iodine was given (after 2 weeks of

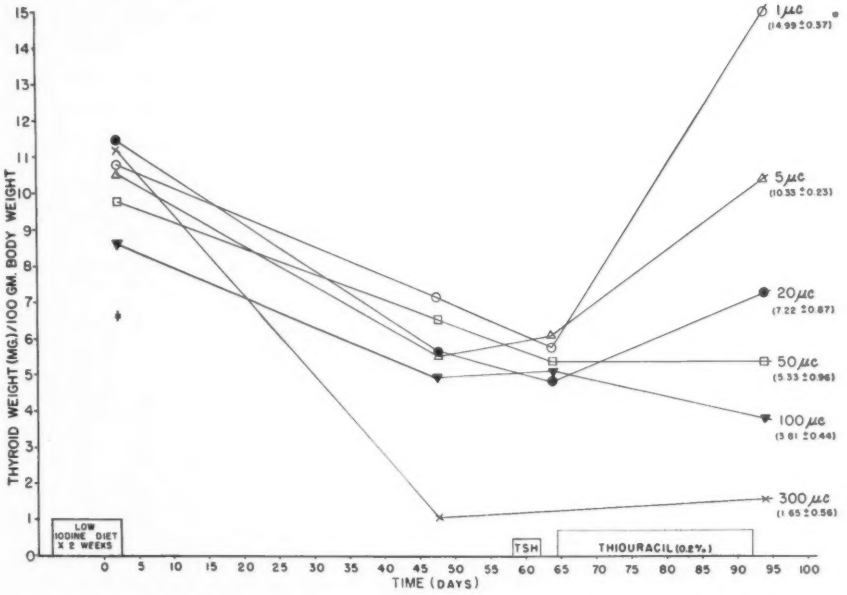


FIG. 5. The response of the irradiated thyroid gland of the rat to the administration of thiouracil as measured by the change in thyroid weight.

* Average thyroid weight of 5 animals and the standard error of the mean.
‡ Thyroid weight of animals on the Purina Fox Chow diet.

the low iodine diet), ranged between 8.47 and 11.48 milligrams per hundred grams of body weight. These thyroid weights were significantly different from those (6.56 mg./100 grams of body weight) obtained from normal control animals that had been given the Purina Fox Chow diet. The decline in the weight of the thyroid, forty-eight days after the administration of the initial dose of radioactive iodine, represents not only the decline resulting from radiation but also a decline which occurred when the low iodine diet was discontinued. A striking decline in the weight of the thyroid was noted in the animals that had received 300 microcuries of radioactive

iodine and was the result of complete destruction of the thyroid by the radiation.

Sixty-four days after the initial dose of radioactive iodine had been given to these animals, at a time when thiouracil administration was instituted, the weights of the thyroid glands of all animals, even those which had received 100 microcuries, were approximately the same (average 4.89–6.08 mg./100 grams of body weight). As a result of the administration of thiouracil, animals which originally had received one microcurie of radioactive iodine developed thyroids which weighed 14.99 milligrams per hundred grams of body weight. The glands of animals which had received 50 microcuries and 100 microcuries demonstrated no capacity to increase in weight. In animals which had received 300 microcuries of radioactive

TABLE 3. THE PITUITARY WIEGHTS OF RATS AT INTERVALS FOLLOWING THE ADMINISTRATION OF VARIOUS DOSES OF RADIOACTIVE IODINE

Dose (microcuries)	2 days after initial dose of I^{131}*	48 days after initial dose of I^{131}	64 days after initial dose of I^{131} and 4 days after TSH	90 days after initial dose of I^{131} and 30 days after thiouracil
1 μc.	5.27 ± 0.46†	3.17 ± 0.48	3.79 ± 0.25	4.70 ± 0.34
5 μc.	3.86 ± 0.42	3.28 ± 0.16	3.46 ± 0.17	5.03 ± 0.34
20 μc.	4.53 ± 0.19	3.17 ± 0.60	2.82 ± 0.31	5.41 ± 0.49
50 μc.	4.98 ± 0.22	3.54 ± 0.52	4.86 ± 0.67	3.48 ± 0.41
100 μc.	4.97 ± 0.59	4.11 ± 0.05	5.44 ± 0.69	3.80 ± 0.24
300 μc.	4.23 ± 0.45	5.70 ± 0.81	5.90 ± 0.42	5.87 ± 0.15
0 μc. and standard diet	4.23 ± 0.48			

* At this time the animals had been on a low iodine diet for the preceding two weeks.
† Weight expressed as milligrams per 100 grams of body weight. Each figure represents an average weight of 5 animals and the standard error of the mean.

iodine, the thyroids were found to be largely replaced by dense fibrous tissue forty-eight days following radiation (see Fig. 10) and in such thyroids hypertrophy would not be expected. As will be subsequently pointed out, animals which had received 5 microcuries of radioactive iodine had no convincing histological evidence of cellular damage before the administration of thiouracil. In spite of this relatively normal appearance of the thyroid, the capacity of such tissue to hypertrophy was significantly impaired.

Changes in pituitary weights during the course of the observations: For comparison with the previously mentioned functional changes in the thyroid, the pituitary glands were removed and weighed at the intervals noted in Fig. 1.

The pituitary weights are recorded in Table 3. There was no significant difference between the pituitary weights of animals that had been given the low iodine diet for two weeks and the normal control animals that had

been given the Purina Fox Chow diet. The pituitary weights of the animals that had received 300 microcuries of radioactive iodine were consistently larger than those of animals that had received smaller doses of radiation, however, these differences were not statistically significant. It is of interest that the pituitary weights in the different groups of animals did not change appreciably following the administration of thiouracil for thirty days.

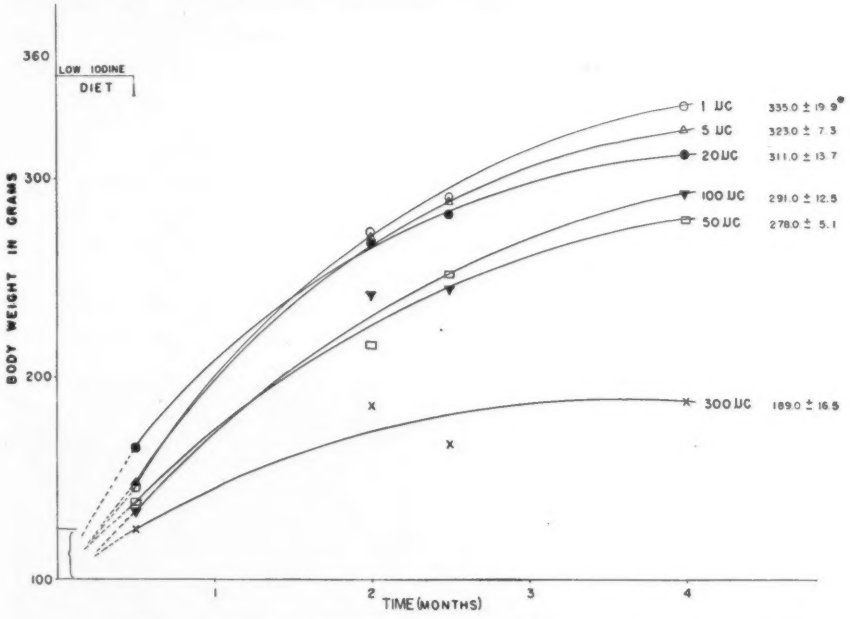


FIG. 6. Changes in body weight in rats that were given various doses of radioactive iodine. A significant impairment in the ability to gain weight was first noted in animals that had received 50 microcuries of the isotope.

* Average body weight of 5 animals and the standard error of the mean.

The effect of radioactive iodine on the growth of rats as measured by body weight and body length: All animals receiving various doses of radioactive iodine were weighed initially and at intervals after the administration of the isotope.

From Fig. 6 it may be seen that the animals did gain weight on this low iodine diet. Control studies revealed that this gain was commensurate with the weight gain of animals on the Purina Fox Chow diet. There was no significant disturbance in the ability to gain weight in animals which had received up to 20 microcuries of radioactive iodine; a slight disturbance in the animals which had received 50 and 100 microcuries; and a marked

disturbance in the animals which had received 300 microcuries. The failure of growth which occurred in the latter group of animals is presumably due primarily to a marked deficiency of thyroid hormone. This will be correlated with the histological damage to the thyroid gland. Measurement of body length revealed that there was a failure in the growth of these animals comparable to the failure in gain of body weight.

It was interesting that animals that had received 20, 50, 100 and 300 microcuries of radioactive iodine exhibited, in a graded sequence, loss of hair especially around the head and rump, coarsening of the hair, and lichenification of the skin about the tail and face.

HISTOLOGICAL STUDIES

As it has been pointed out, one lobe of the thyroid was preserved for histologic preparation when sample animals were sacrificed after a series of observations. Observations were made on five or six animals from the different irradiated groups in each instance.

Two days after I^{131} administration: The thyroids of the series of animals that were sacrificed 2 days after the administration of 1, 5, 20, 50 and 100

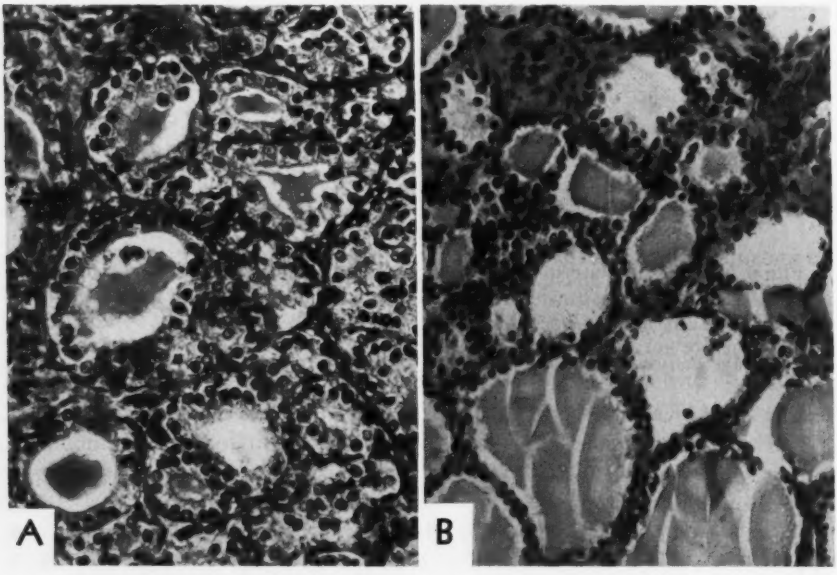


FIG. 7A. Thyroid gland from a rat sacrificed 14 days after being on a low iodine diet and 2 days after the injection of 1 microcurie of radioactive iodine. Note the significant degree of stimulation. ×270.

FIG. 7B. Thyroid gland from a control rat maintained on an adequate iodine intake (Purina Fox Chow). ×270.

microcuries of radioactive iodine uniformly exhibited a moderate degree of stimulation induced by the low iodine diet which had been given during the preceding two weeks. These glands showed acini of variable size, lined with uniformly large cells with clear vacuolated cytoplasm, and centrally located, round and regular nuclei (See Fig. 7A). The histological appearance of these thyroids may be contrasted with those of normal control animals maintained on an adequate iodine intake (Purina Fox Chow). Here there was little to no evidence of stimulation (See Fig. 7B).

The thyroids of animals which had received the 300 microcurie dose showed changes which were principally in the central portion, and were

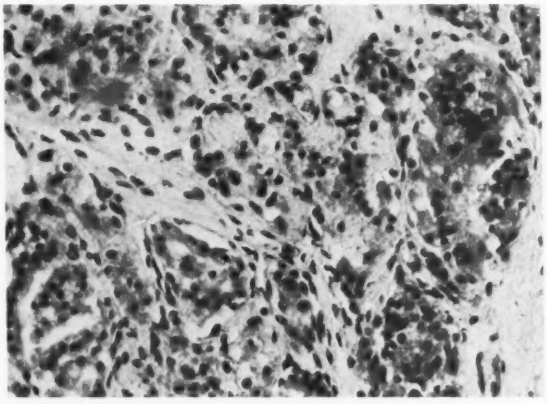


FIG. 8. Thyroid gland from a rat 2 days after the administration of 300 microcuries of radioactive iodine. Observe the disorderly follicular architecture, desquamation of cells into acinar lumens, and edema of the connective tissue stroma with scattered inflammatory cells. ×270.

characterized by: a loss of normal follicular architecture, desquamation of cells into tissue spaces and acinar lumens, swelling of the cellular cytoplasm, pycnosis of nuclei, edema of the stroma, and infiltration of the latter with inflammatory cells (See Fig. 8).

Forty-eight days after I^{131} administration: Sample animals were sacrificed from each of the groups 48 days after the various doses of radioactive iodine had been administered, and 46 days after the animals had been returned to the Purina Fox Chow diet.

In spite of the adequate iodine intake, thyroidal stimulation was still observed in animals that had received one microcurie of the isotope (See Fig. 9A). However, in the thyroids of animals that had received 5, 20, 50, and 100 microcuries, an increase in cell height with lack of colloid was present to an even greater degree (See Fig. 9B) than in the thyroids of

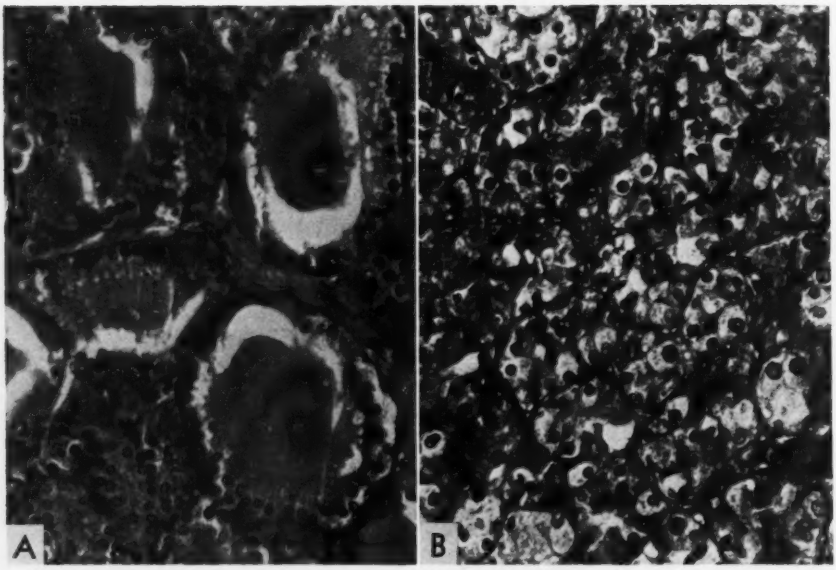


FIG. 9A. Thyroid gland from a rat sacrificed 48 days following the administration of 1 microcurie of radioactive iodine and 46 days after having been returned to an adequate iodine intake. Stimulation is still evident at this interval but is somewhat less than that noted at 2 days after radiation. Compare with Fig. 7a. ×270.

FIG. 9B. Thyroid gland from a rat sacrificed 48 days after the administration of 50 microcuries of radioactive iodine. Note the marked degree of cellular hypertrophy despite the resumption of an adequate iodine intake. Compare with Fig. 9a. ×270.

animals that had received one microcurie of radioactive iodine. The cellular hypertrophy was present to an even greater degree than that which had been observed in all animals after having been on the low iodine diet for 14 days, but was not associated with an increase in the weight of the thyroid at this interval of time (See Fig. 5). This apparently paradoxical observation will be discussed later.

It was very difficult to identify definite histological features which reflected the effect of radiation in the thyroids of animals which had received up to 20 microcuries. Among the animals which received 20 microcuries, however, there was a rare nucleus which was somewhat larger than the others, a finding which was not observed in animals which received one and five microcuries of radioactive iodine.

In the thyroids of animals that had received 50 microcuries, definite variation in nuclear size was noted. Cells which contained slightly enlarged nuclei occurred in a totally random fashion and were identified only after

carefully scanning the entire gland. These nuclei were unusual only as to size, presenting normal outlines and normal chromatin patterns. In the animals that had received 100 microcuries, the variation in nuclear size was more conspicuous and occasionally nuclei were found which were decidely irregular in shape. Some of the thyroids in this group contained an increased amount of connective tissue scattered throughout the stroma.

In the animals that had received 300 microcuries, the thyroid was virtually obliterated and replaced by dense scar tissue (See Fig. 10). However, scattered through this fibrous tissue were occasional clusters of irregular hyperchromatic nuclei which in some instances were not accompanied by

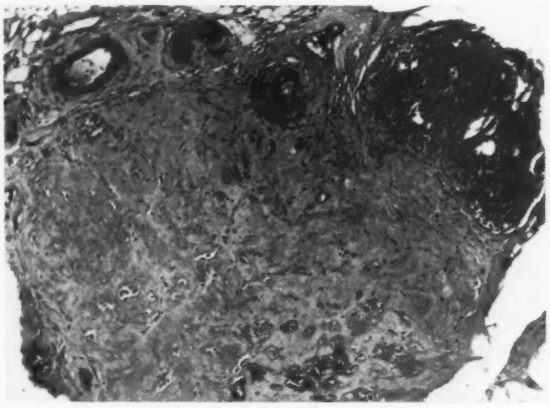


FIG. 10. Thyroid gland of a rat 48 days after the administration of 300 microcuries of radioactive iodine. Note the marked fibrous replacement of the thyroid and the swelling of the walls of the two arteries at the top center of the photomicrograph. A parathyroid gland in the right upper corner appears essentially normal. ×60.

any discernible cytoplasm. Practically no follicular structures remained. Scattered here and there throughout the dense hyalinized tissue were clumps of hemosiderin.

Although small arteries and veins showed signs of swelling of their walls in the animals that had received 100 microcuries of radioactive iodine, vascular changes were more impressive in the animals that had received 300 microcuries. Here the vessels showed an obliteration of the delineation between muscular and endothelial layers by replacement with an eosinophilic acellular substance. Actual evidence of thrombosis was only rarely observed.

The parathyroid glands studied in these animals revealed a considerable resistance to radiation; for even in the rats which had received 300 microcuries where there was complete destruction of the thyroid, the para-

thyroids remained essentially normal. There was, however, a minimal degree of fibrosis about the periphery of the parathyroids and an accentuation of the fibrous stroma (See Fig. 10).

Sixty-seven days after I^{131} administration: Sixty-seven days after the administration of various doses of radioactive iodine and 3 days after the discontinuation of the TSH injections (daily for 4 days), some animals were sacrificed.

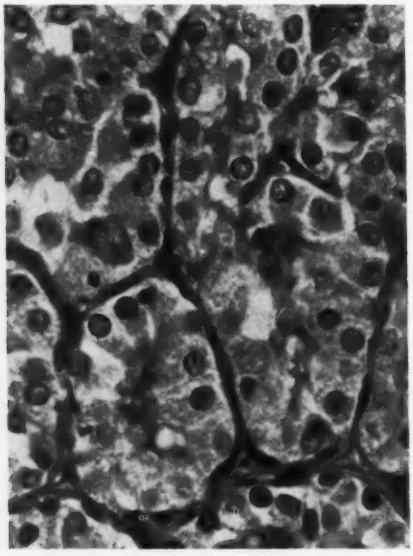


FIG. 11. The thyroid of a rat 94 days after the administration of 1 microcurie of radioactive iodine and following a 30-day course of thiouracil. Observe the cellular hypertrophy and uniform nuclei. ×520.

Consistent with the previously described lack of apparent physiological response to TSH, these thyroid glands failed to exhibit any more evidence of stimulation. An explanation for this lack of response to TSH has been previously proposed.

Histologically, the thyroid glands of animals that had received 1 microcurie of radioactive iodine showed less evidence of stimulation than had been seen in this group of animals after having been on the low iodine diet for two weeks. However, in the animals that had received 5, 20, 50, and 100 microcuries, the increase in height of the thyroid cells as noted and described at the 48-day interval was still an impressive histological feature, and in all instances was much more marked than in the thyroids of animals that had received one microcurie of the isotope.

In the thyroid glands of the animals that had received 5 microcuries, no abnormalities could be detected except for the cellular hypertrophy. This observation was also true for the animals that had received 20 microcuries except for the presence of scattered, enlarged nuclei. In the thyroids of the rats that had received 50 and 100 microcuries, variations in nuclear size were slightly more prominent than had been seen at the 48-day interval. In addition, occasional small foci of fibrosis were noted in the latter groups principally in the form of an increase in the connective tissue stroma in the central portions of the thyroids.

Ninety-four days after I^{131} administration: Ninety-four days after various doses of radioactive iodine had been administered, and at the completion of the 30 days on thiouracil (0.2 per cent solution as drinking water), additional histological observations were made.

The thyroids of animals which had received 1 microcurie of radioactive iodine showed no abnormalities other than a marked degree of stimulation (See Fig. 11). In the thyroids of animals that had received 5, 20, 50, and 100 microcuries of radioactive iodine, evidence of stimulation was very prominent but this had been observed even before the administration of thiouracil, though to a lesser degree.

In the thyroid glands of animals which had received 5 microcuries of radioactive iodine, variations in nuclear size were seen that had not been present before the administration of thiouracil. It appears that thiouracil administration made evident not only functional changes (See Fig. 5), but histological changes in this group of animals. In the animals that had received 20, 50, and 100 microcuries of the isotope, nuclear variations were more numerous than had been seen at the 67-day interval. In particular, the latter two groups exhibited many bizarre and atypical nuclei. These were most often abnormalities in nuclear size and outline, with a tendency to be several times the normal or average. The majority of these abnormal nuclear forms presented no distinguishable alterations from a normal chromatin pattern, but occasional ones, especially in the animals that had received 100 microcuries, exhibited a homogeneous hyperchromatism. It was interesting that the abnormal nuclei were scattered about in no definite arrangement, being present at times in only one or two cells of a given follicle. It appears that the administration of thiouracil and not just the passage of time accentuated these nuclear changes (See Fig. 12A and 12B).

Here in the animals that had received 300 microcuries, practically total replacement of all parenchyma by dense fibrosis was seen with only the parathyroid remaining as a recognizable structure.

One year after I^{131} administration: One year after the administration of radioactive iodine, sample animals from each group were given a tracer

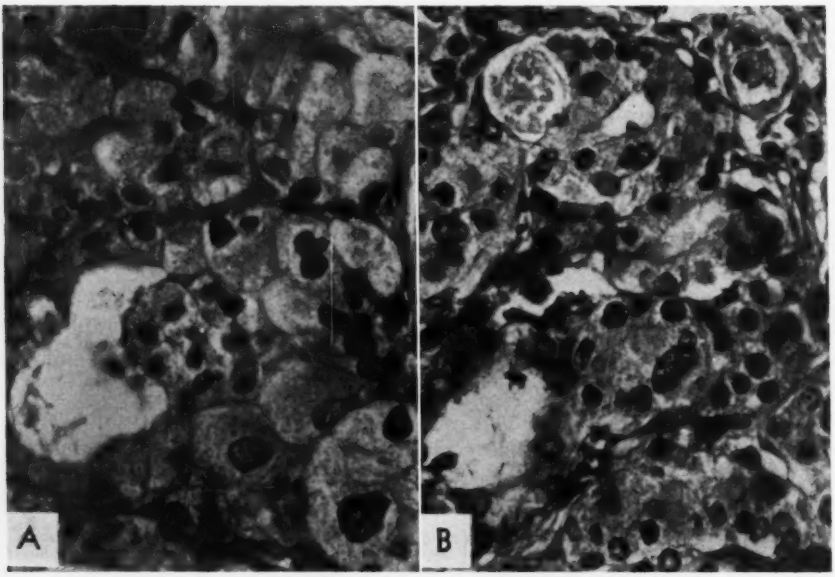


FIG. 12 A & B. The thyroids of 2 rats, 94 days following the administration of 100 microcuries of I^{131}. One animal (Fig. A) also received thiouracil during the last 30 days prior to being sacrificed, while the second rat (Fig. B) was given no thiouracil. Note the more marked nuclear irregularities in the thyroid of the rat which received thiouracil. ×520.

dose of the isotope (See Fig. 1) and 2 days later these animals were killed with ether and the thyroid glands were removed en bloc with the trachea for histological and autoradiographic studies.

In the animals that had received 1 microcurie, the thyroid histology was quite normal.

In the thyroids of rats that had received 5 microcuries, the cellular hypertrophy was as impressive as at the 94-day interval following a course of thiouracil. In addition, the swollen cytoplasm of the cells contained finely granular, slightly eosinophilic material. The nuclei varied in size from a slight to moderate degree.

The thyroids of animals that had received 20, 50, and 100 microcuries likewise exhibited a persistence of the impressive cellular hypertrophy noted in the previous observations. By this time these animals had been maintained on an adequate iodine intake for one year. The individual follicles showed a considerable variation in size and contained only sparse amounts of colloid. The plump cells of the acini revealed eosinophilic granular cytoplasm in contrast to the clear foamy appearance observed

at the 94-day interval (See Fig. 13). The nuclei exhibited a multiplicity of atypical and bizarre forms, more pronounced in a graded sequence in the thyroids of animals that had received 20 to 100 microcuries of radioactive iodine. Radioautographs made from the thyroid sections of these one year post-radiation animals and 2 days after the administration of a tracer dose of I^{131} disclosed a fairly marked blackening of the film even in the animals that had received 100 microcuries (See Fig. 14A & B), thus indicating a capacity to take up iodine in spite of diffuse, marked cellular changes.

At the end of a year, the thyroids of the animals that had received 300 microcuries of radioactive iodine were replaced by dense scar tissue with hardly a vestige of gland to be found. The parathyroid glands at this time showed some stromal fibrosis, particularly at the edges, but were generally quite intact.

The other perithyroidal structures in the rats that had received 100 and 300 microcuries showed no impressive histologic alterations except for the small blood vessels. The walls of the arterioles often exhibited eosinophilic degenerative changes, but frank thrombosis was unusual. Evidence of significant tracheal damage was unimpressive, for in all groups of animals

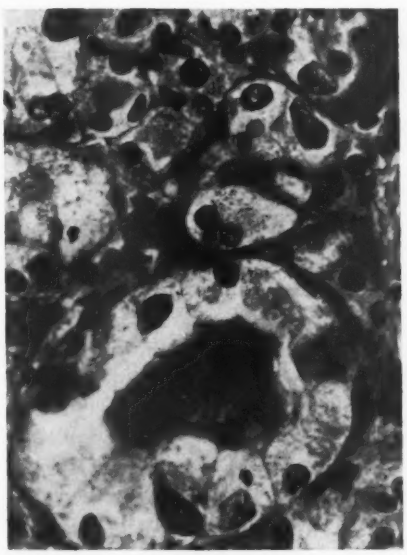


FIG. 13. The thyroid gland of a rat sacrificed 1 year after receiving 100 microcuries of I^{131}. In addition to the nuclear variations, note the persistent cellular hypertrophy despite maintainance of the animal on an adequate iodine intake. Cytoplasmic granularity (eosinophilic) is apparent in some of the larger cells. ×520.

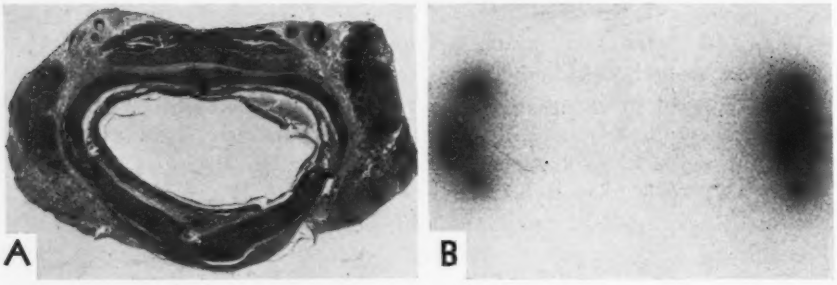


FIG. 14 A & B. Photomicrograph (A) and autoradiograph (B) of a section through the neck (at the level of the thyroid) of a rat sacrificed one year following the administration of 100 microcuries of I^{131}. The animal had received 25 microcuries of the isotope 2 days before being sacrificed. Note the diffuse darkening of the autoradiograph corresponding to the thyroid lobes. The histologic structure of the thyroid at a higher magnification is illustrated in Fig. 13. ×13.

at the termination of a year following radiation, the tracheal epithelium was still preserved with demonstrable cilia.

One and one-half years following I^{131} administration: One and one-half years after the administration of various doses of radioactive iodine, several animals from each group were sacrificed.

The thyroidal histology was essentially as described at the one year interval following radiation. However, a small adenoma of the thyroid was found in one of the animals that had received 5 microcuries of radioactive iodine. This was the only adenoma that was seen in the entire group of 500 animals.

No parathyroid tumors were found in these animals.

DISCUSSION

Observations have been made on the effects of internal radiation from radioactive iodine on various functions of the thyroid and have been correlated with concurrent histological changes. The rate of loss of radioactive iodine from the thyroid has been shown to be roughly proportional to the magnitude of the dose (See Fig. 2); however, it was not until 300 microcuries (288,000 rep) were given that the loss of the isotope from the gland was strikingly increased. Extensive morphological damage was observed only in the glands which had received this large dose of the isotope. Of considerable more significance were the subtle morphological and functional changes that resulted from sublethal doses of radiation to the thyroid.

Although histological changes caused by radiation in the thyroid were not evident two months after the administration of 5 microcuries of radio-

active iodine (5,800 rep), certain functions of the thyroid were significantly impaired as shown by a decreased capacity of the thyroid: 1) To take up iodine (Fig. 3); 2) To hypertrophy in response to a low iodine intake (Fig. 4); and 3) To hypertrophy in response to the administration of thiouracil (Fig. 5). These observations suggest that functional changes were apparent before there were recognizable histological effects of radiation. In the thyroids of animals which had received 20 (30,000 rep), 50 (80,000 rep), and 100 (197,000 rep) microcuries, histological changes were present in two months, and, in general, the alterations in function and morphology bore a quantitative relationship to the magnitude of the dose.

Moderate impairment in growth (body weight and length) was observed two months after the administration of radioactive iodine in the animals which received 50 and 100 microcuries. The impairment was marked in the animals which had received 300 microcuries. This is probably additional evidence of thyroid insufficiency.

A persistent increase in the height of thyroid epithelium was found 18 months after 5, 20, 50, and 100 microcuries of radioactive iodine had been given. This occurred in spite of an adequate iodine intake during this interval of time. It is interesting that the increase in the height of the thyroid epithelium was present in the animals which had received only 5 microcuries of radioactive iodine at a time when no other histological abnormalities were apparent.

Since the irradiated thyroids had a reduced functional capacity, it is logical to assume that the increase in cell height resulted, in part, from the stimulus caused by a deficiency in the production of thyroid hormone. In spite of the marked and persistent hypertrophy of the cells, the thyroids failed to increase in weight. Two obvious morphological features of stimulation in the thyroid are: 1) An increase in the height of the epithelium (cellular hypertrophy), and 2) Cellular proliferation which results in hypertrophy of the thyroid gland. When thiouracil was given to these animals, the cellular hypertrophy became more pronounced. In spite of this increase in the height of the cells, the thyroids failed to increase in weight proportionately to the amount of radiation received. The observations suggested that the mechanism of cellular division was impaired while the mechanism of cellular hypertrophy was not. Eckert, Probstein and Galinson (1937) irradiated the thyroids of guinea pigs with 50–400 mg.-hour of radium at 0.5 cm. and found that 100–300 mg.-hour would impair the ability of the thyroid parenchyma to reproduce. Lea (1947) states that "An increase in the size of cells, or of their nuclei, following irradiation has been observed in a number of materials. In some cases, though perhaps not in all, the effect requires no explanation other than the inhibition of division which is known to occur. If the cells are prevented from dividing, but

continue to grow at the normal rate, then the size of the cells will increase instead of their number."

With the passage of time, irregular nuclei of variable size appear in thyroids which had received moderate or large doses of radiation. Following a small dose of radiation (5 microcuries) these bizarre nuclear changes were not apparent. However, when the irradiated thyroids were stimulated by the administration of thiouracil, the bizarre nuclear changes became manifest in the thyroids of animals that had received 5 microcuries and were exaggerated in those of animals that had received larger doses of radiation. It thus appears that marked stimulation of the irradiated thyroid by the administration of thiouracil makes apparent or accentuates cellular changes produced by radioactive iodine.

An interesting consideration arises concerning the nature of the large, irregular nuclei. If the hypothesis of failure of cellular division is true, then these bizarre nuclear forms may represent an increase in the nuclear contents preparatory to cellular division which is ultimately thwarted in some manner. Whether these bizarre nuclear patterns bear any relationship to those of neoplastic disease is not clear.

In further support of the concept that radiation impairs the normal sequence of cellular reproduction was the paucity of either regeneration or adenoma formation in any of the groups of animals one and one-half years following the radiation.

The persistence of increased cell height is probably due to the lack of compensatory regeneration in the irradiated thyroids. Significant regeneration would probably have caused a lessening if not a disappearance of the cellular hypertrophy.

Only one clearly identifiable adenoma was found in the entire series of 500 animals, a finding which is contrary to the observations of some other authors (Doniach, 1950; Goldberg and Chaikoff, 1951) who described an increased incidence in the formation of adenomas of the thyroid after radioactive iodine administration. Apart from the formation of adenomas, the question of regeneration in the irradiated thyroid is highly important. It would seem that if a significant degree of regeneration had occurred in these irradiated thyroids, the normal compensatory mechanism would have caused a decrease in the persistent cellular hypertrophy, which was observed shortly after the radiation. It should be mentioned that regeneration per se, without adenoma formation, is not easily recognized in the thyroid since the morphological criteria for cellular regeneration are quite non specific and ill defined. In spite of this, it seems unlikely that regeneration could have accounted for a significant portion of these irradiated thyroids, because the histological manifestations of radiation were quite diffuse in nature and regenerated zones would be expected to stand out as relatively

normal undamaged tissue, unless the regenerated tissue appeared in a mutant form.

SUMMARY

Various single doses of radioactive iodine ranging from 1 to 300 microcuries were administered to a total of 500 rats. Concurrent functional and histological studies were made on sample animals at intervals ranging from two days to eighteen months after the administration of this isotope.

Though where was no histological evidence of radiation damage in the thyroids of animals which had received 5 microcuries of radioactive iodine (5,800 rep), there was definite impairment of function. These disturbances of thyroid function occurred in a graded sequence in the animals that had received 20, 50, and 100 microcuries of radioactive iodine. No functional abnormalities were noted in animals that had received 1 microcurie of the isotope.

Two of the most interesting histological changes following radiation were: a persistent increase in cell height, and bizarre nuclear changes. The former was present for one and one-half years following radiation in spite of an adequate iodine intake in animals that had received 5, 20, 50, and 100 microcuries of radioactive iodine. The bizarre nuclear changes became apparent only after the administration of thiouracil in animals that had received 5 microcuries and were accentuated in animals that had received 20, 50, and 100 microcuries of radioactive iodine. The failure of the thyroid to enlarge in response to the administration of thiouracil in spite of the persistent hypertrophy of the cells suggests that cellular division was impaired by the radiation.

Only one adenoma of the thyroid was seen in these 500 animals and this occurred in an animal one and one-half years after the administration of 5 microcuries of radioactive iodine.

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PRODUCTION OF CORTICOIDS BY RAT ADRENALS *IN VITRO*

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THE isolated adrenal cortex can be stimulated by the *in vitro* addition of corticotrophin. Tepperman (1950), using dog adrenal cortex slices, and Saffran, Bayliss and Webb (1951), using rat adrenal glands, found that corticotrophin, added *in vitro*, caused a fall in adrenal ascorbic acid and an increase in adrenal respiration. Pincus, Hechter and Zaffaroni (1951) and Vogt (1951) showed that the addition of corticotrophin to the perfusing medium increased the production of corticoids by perfused adrenal glands of cattle and dogs.

This paper describes the formation of corticoids by rat adrenal glands *in vitro* and the stimulation of this formation by the addition *in vitro* of corticotrophin.

METHODS

The adrenal glands of young adult male Sprague-Dawley or Long-Evans rats were removed under nembutal anesthesia, dissected free of fat, and bisected with scissors. The glands were placed into Warburg flasks containing 2 ml. of Krebs-Ringer-phosphate medium (Dixon, 1943) with added glucose (200 mg. %). The side-bulbs of all flasks contained 0.1 ml. of a solution of corticotrophin (ACTHAR, Armour) in the medium. Each rat contributed one adrenal to the control flask and one to the experimental flask. The incubations were in an atmosphere of oxygen at 38°, except where noted. The corticotrophin was tipped into the experimental flask at the beginning of the incubation period, and into the control flasks at the end of the period. After an incubation period of two hours the corticoids in the medium were estimated by the bioassay method of Speirs and Meyer (1951) and by a spectrophotometric method.

For the bioassay, groups of 4 or more mice were injected with 0.1 to 0.25 ml. aliquots of the medium from each flask and the amount of corticoids present was calculated from the mean percent decrease in the circulating eosinophils. Our calibration curve, relating the per cent fall in eosinophils with the dose of cortisone, was practically identical with that of Speirs and Meyer for cortisone acetate (1951) (Fig. 1).

The remainder of the medium was transferred to an extraction tube with the help of two 1 ml. rinses with water. The extraction tubes were made by sealing the non-ground ends of the outer members of 24/40 ground joints. Ten ml. of twice-distilled chloroform were added to the tube. The tube was stoppered with a ground-glass stopper and was shaken 100 times by hand. The stopper was removed and rinsed into the tube with a stream of chloroform from a wash bottle. The tube was centrifuged at a moderate speed for 5 minutes. The spinning separated the phases cleanly with a layer of protein at the

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interface. The water layer was aspirated off with a fine-tipped pipette and was discarded. Enough sodium sulfate was added to the chloroform to remove all the remaining water. The tube was again centrifuged for 5 minutes. The sodium sulfate packed down in the bottom of the tube, carrying the protein with it. The chloroform extract was carefully decanted into another, dry, extraction tube. The sides of the first tube and the sodium sulfate were washed 3 times with a stream of chloroform *without* re-suspending the sodium sulfate. The washings were added to the second tube. A boiling stone was added to the tube and the chloroform extract was taken to dryness at about 50° with a vacuum from a water pump. The last traces of chloroform were removed with a mechanical pump at

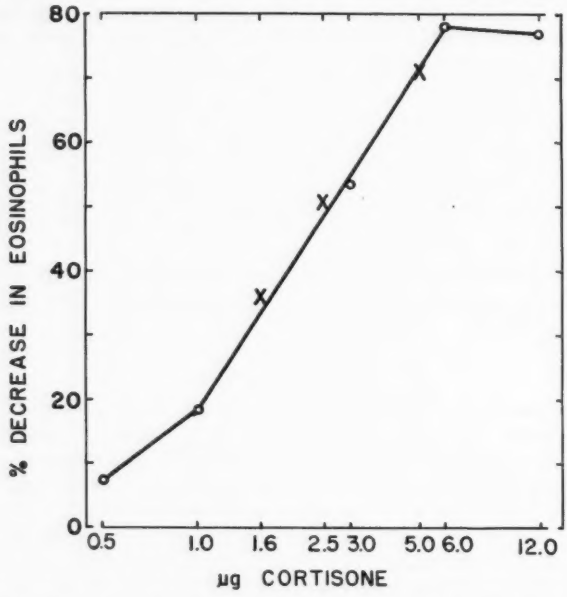

FIG. 1. Calibration curve for the estimation of corticoids by the bioassay method of Speirs and Meyer (1951). The circles are the values obtained by Speirs and Meyer (1951) for cortisone acetate, and the crosses are the values obtained in our laboratory for cortisone.

50° for 1 hour. The chloroform was removed completely because it interfered in the spectrophotometric determination. The residue was dissolved in 4 ml. of reagent grade methanol and 3 ml. were transferred to a quartz cuvette of a Beckman spectrophotometer. The optical densities at 225, 240, and 255 mμ were determined against a methanol blank. The absorption at 240 mμ. caused by the α, β-unsaturated ketonic grouping was calculated by the method of Allen (1950):

$$\text{calculated optical density}_{240} = \text{OD}_{240} - \frac{\text{OD}_{225} + \text{OD}_{255}}{2}.$$

The amount of corticoids was read from a graph constructed by plotting the calculated optical density against concentration for pure solutions of cortisone in methanol, con-

TABLE 1. COMPARISON OF RESULTS OBTAINED BY THE BIOASSAY AND SPECTROPHOTOMETRIC METHODS

Bioassay μg.	Spectrophotometry corticoids/100 mg.
9.8	0
15.0	11.1
35.5	37.0
47.5	32.5
52.5	40.0

Correlation coefficient, $r = 0.936$
$0.02 > P > 0.01$

taining 2 to 12 μg. per ml. The recoveries of added cortisone from aqueous solution averaged $64 \pm 1.4\%$ in 14 determinations.

There was a significant correlation between the results obtained by the bioassay and by the spectrophotometric method (Table 1) so that in many experiments only the ultraviolet absorption measurements were made.

All results are expressed as μg. of cortisone per 100 mg. of fresh weight of adrenal tissue. The corticotrophin is expressed as mg. of the provisional standard preparation, La-1-A.

TABLE 2. INCREASED PRODUCTION OF CORTICOIDS IN THE PRESENCE OF CORTICOTROPHIN

Strain of rat	Corticotrophin added	No. of adrenals	Corticoids found	
			Control	Corticotrophin
	μg./ml.		μg./100 mg./2 hrs.	
Sprague-Dawley	100	2	0*	24.4
	100	5	5.0	14.3
	100	5	7.1	17.3
	100	10	8.8	13.0
	150	4	0	21.1
	150	4	0	19.8
	250	4	0	26.2
	1000	8	0	37.0
Long-Evans	1	8	5.7	7.1
	10	8	7.4	7.0
	150	4	6.7	11.4
	250	4	7.8	25.8
	1000	10	11.1 (15.0)	32.5 (47.5)

* The corticoids were estimated by the spectrophotometric method. The figures in parentheses were obtained by the Speirs-Meyer bioassay.

RESULTS

In 21 experiments in which rat adrenals were incubated at 38° for 2 hours, the medium contained an average of 5.0 ± 0.7 (S.E.) μg. of corticoids per 100 mg. of tissue. The addition of corticotrophin increased the amount of corticoid obtained (Table 2); the amount of the increase depended upon the amount of corticotrophin added (Fig. 2).

The effect of corticotrophin was absent at 2° (Table 3). In these experiments the medium and the glands were analyzed together. The results

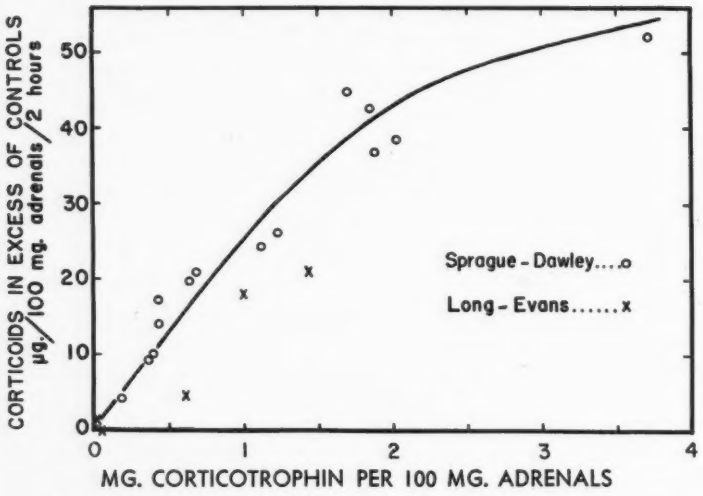

FIG. 2. Relation between amount of corticotrophin and production of corticoids by adrenal glands of the Sprague-Dawley and Long-Evans strains of rats. All determinations were by the spectrophotometric method.

were of the same order of magnitude as those obtained by the analysis of the medium alone in the other experiments.

According to the spectrophotometric test no corticoids were produced by testis or liver tissue of the rat with or without added corticotrophin.

TABLE 3. PRODUCTION OF CORTICOIDS AT 2° AND AT 38°

Corticotrophin added	Corticoids found	
	2°	38°
µg./ml.	µg./100 mg./2 hrs.	
0	4.7*	6.4
1000	0	52.5 (40.0)
1000	9.8 (0)	35.5 (37.0)

* The corticoids were determined by the Speirs-Meyer bioassay. The figures in parentheses were obtained by the spectrophotometric method.

DISCUSSION

In this investigation corticoids were estimated by the Speirs-Meyer bioassay and by the absorption of light at 240 mµ by chloroform-soluble substances. The Speirs-Meyer method is specific for the C_{11}-oxygenated corticoids (Speirs and Meyer, 1951). The spectrophotometric method, although not specific for these compounds, also appears to measure corticoids because the same adrenal extracts gave similar results by both methods (Table 1). This is to be expected because the biologically-active adrenal

steroids are chloroform-soluble and have an α,β-unsaturated ketone which absorbs light at 240 mμ. Other compounds with these physical properties, such as testosterone and progesterone, are not active in the Speirs-Meyer assay and are probably not formed in appreciable amounts in the adrenal gland. Furthermore, testis and liver tissue, with or without added corticotrophin, gave negative results in the spectrophotometric tests.

The relationship between the amount of corticotrophin added and the formation of corticoids is being investigated as an assay for corticotrophin.

Only relatively small amounts of corticoids were found in both gland and medium kept at 2°. Therefore, there are no large stores of preformed corticoids in the adrenal. Corticotrophin apparently acts by stimulating the formation of corticoids from precursors, rather than promoting the release of corticoids from the gland.

SUMMARY

Substances produced by the rat adrenal gland *in vitro* show the following properties: (a) they are soluble in chloroform, (b) they absorb light strongly at 240 mμ, (c) they cause a decrease in the number of circulating eosinophils in adrenalectomized mice, (d) their formation is stimulated by corticotrophin, and (e) they are not produced by testis or liver tissue. Therefore, we conclude that the isolated rat adrenal produces corticoids.

The amount of corticoids produced depends upon the amount of corticotrophin added to the adrenal *in vitro*.

Acknowledgements

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We wish to thank Dr. R. S. Speirs for invaluable assistance in setting up the Speirs-Meyer bioassay. The able technical assistance of Mrs. Olga Sher and Miss Barbara Ronalds is gratefully acknowledged.

We are indebted to Dr. R. A. Cleghorn for his encouragement and advice.

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THE EFFECTS OF PROLONGED INFUSION OF THE DOG'S PANCREAS WITH GLUCOSE

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SEVERE hydropic degeneration of the islands of Langerhans and permanent diabetes have been produced in cats by repeated intraperitoneal injections of glucose (Dohan and Lukens, 1948). The prolonged, sustained hyperglycemia so produced was thought to be the most important factor in the production of the hydropic change and the persistent diabetes. To test further this possibility it seemed desirable to localize a sustained hyperglycemia to the pancreas or a portion of it. In this way the responses to systemic hyperglycemia might be avoided and changes found in the infused organ might be attributed to the local hyperglycemia. In the experiments reported herein, the successful infusion of a part of the pancrease with glucose usually resulted in systemic hypoglycemia and in changes in the islands of Langerhans which includes both hyperplasia and hydropic degeneration. Diabetes did not occur, nor was it expected, since only part of the pancreas was infused.

The literature on the effect of the administration of glucose upon the pancreas may be placed in one of two categories. The first, *viz.* the administration of glucose by the oral, intravenous or other parenteral routes, has been reviewed (Dohan and Lukens, 1948; Barron and State, 1949) and it is only necessary to add the subsequent studies of Haist *et al.* (1949) and Vining (1949). This has been reviewed (Dohan and Lukens, 1948; Barron and State, 1949) and it is only necessary to add the subsequent studies of Haist *et al.* (1949) and Vining (1949). Haist *et al.* (1949) reported the effect of glucose on island growth and hypertrophy by the direct measurement of island volume in rats. The importance of glucose was emphasized by the occurrence of hypertrophy and hyperplasia even in hypophysectomized rats. The results of Vining (1949) who used prolonged intravenous infusion will be considered in discussion.

The second group of studies are those in which infusion of the arterial cir-

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culation of the pancreas has been performed for relatively short periods. These have been summarized by Kosaka (1933), Foglia and Fernandez (1936) and by Soskin and Levine (1946). This type of experiment will be discussed with the results of our experience in arterial infusion.

METHODS

The perfusion apparatus was slightly modified from that developed by Stengel and Vars (1939) as further improved by Rhode *et al.* (1949). These articles describe the perfusion apparatus, the method of attaching the tubing to the dogs' harness and so forth. Further information on the use of plastic catheters is given by Last, Pitesky and Marbarger (1950).

Insertion and fixation of the arterial perfusion tube. The introduction of the polyethylene tubing into the artery was accomplished under pentobarbital anesthesia, through a long mid-line incision. For anatomical reasons, female beagle-type dogs of about 10 kg. body weight were found most suitable for intubation of arteries supplying the pancreas. Prior to operation all the apparatus was tested and the guard tubing and perfusion tubing properly attached to the dog's harness. After opening the abdomen the perfusion tubing was brought through the left lateral abdominal wall. This was done with a sharpened hemostat. A small puncture wound was made by pressure from within the abdomen and the tip of the perfusion tube grasped and pulled through. The guard or sleeve tubing was placed to protect the perfusion tubing up to the site of the puncture

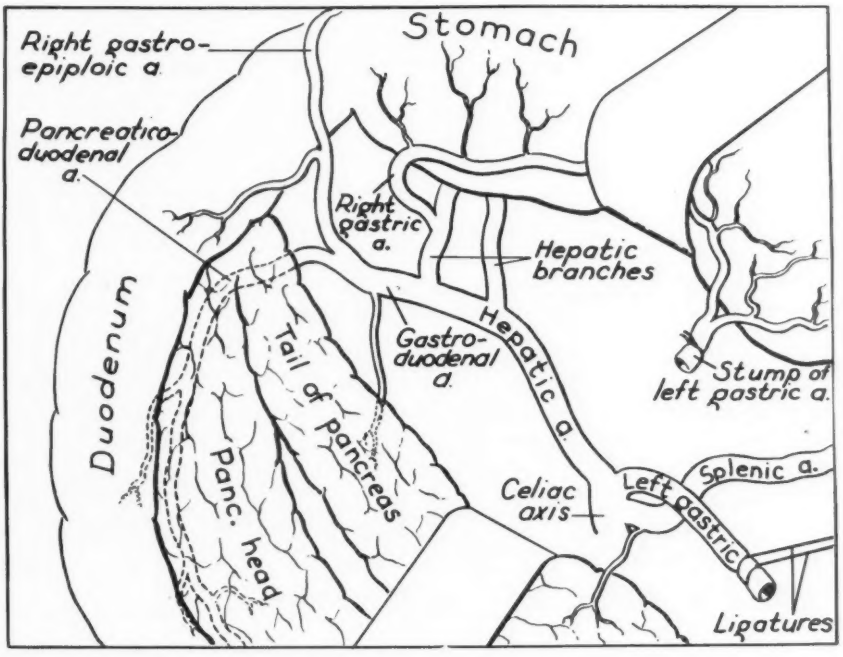

FIG. 1. The celiac axis and some of its branches in the dog.

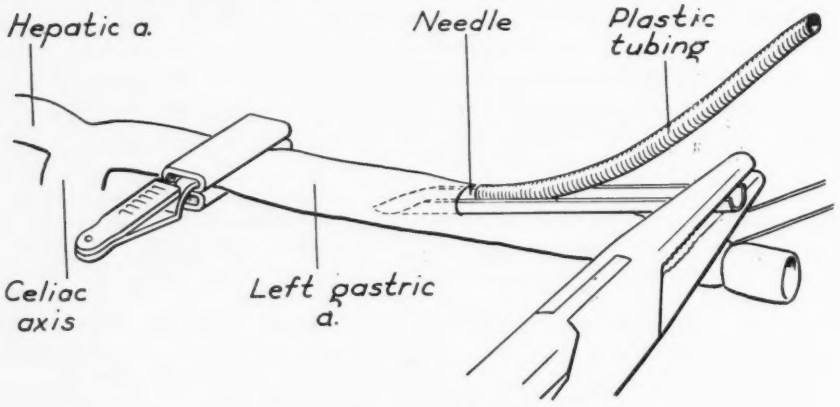

FIG. 2. Diagram of insertion of tubing in artery.

wound. The guard tubing was sewed to the skin at appropriate intervals until the harness was reached.

Figure 1 shows the anatomy of this region and Figures 2 and 3 show the technic of inserting the tube into the hepatic artery. It was found that if the tube was inserted into a branch of the artery to be perfused, and threaded from it into position, less hemorrhage and thrombosis occurred than when the tube was placed directly in the artery to be infused. In the case of the hepatic artery the left gastric artery was used for insertion and the tube was then threaded across the celiac axis and into the hepatic artery. In order to do this the left gastric artery was doubly ligated and cut close to its bifurcation. The proximal stump, about 2 cm. in length, was freed from the vein and celiac plexus sufficiently to permit its alignment with the hepatic artery (Figs. 1 and 2). Alignment and insertion were facilitated by means of moderate tension on the ligatures attached to the end of the left gastric artery. Insertion into the artery was accomplished by means of a suitable intravenous needle, ground so that it served as a sharp "grooved director." The

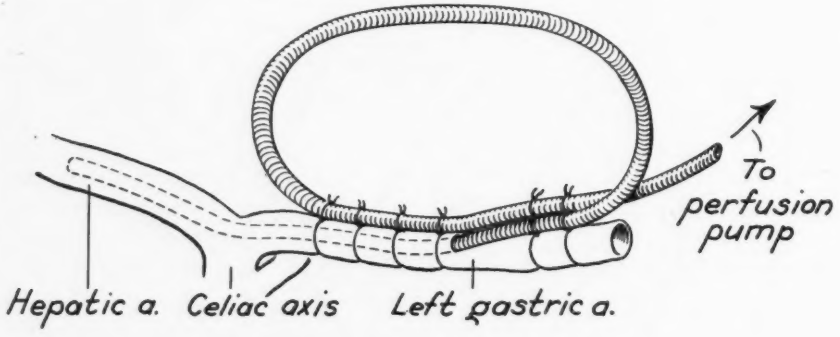

FIG. 3. Final position of tubing in artery.

depth of the groove was slightly more than half the diameter of the tube to be inserted. A rubber-guarded bulldog clamp was placed at the base of the freed left gastric stump just before the insertion of the tube. If left on for more than a few minutes, spasm was likely to occur. The "grooved director" was held in a needle holder and inserted for about 0.5 cm. into the lumen of the artery (Fig. 2). An assistant using forceps then threaded the tubing along the groove and into the lumen of the artery. The tube was then marked by a single overhand knot of black silk so that the amount inserted could be estimated. After the insertion of the tube, it was held in place by grasping it with forceps close to the site of entry into the artery and the "grooved director" was then removed. The bulldog clamp was then removed, perfusion with physiological saline started, the arteries aligned as noted above, and the tube passed across the celiac axis and into the hepatic artery (Fig. 3). With good alignment this could be done on the first try. After passage of the tip of the tube to the desired site the bulldog clamp was replaced on the stump of the left gastric artery and the tube thus held in place while three to five firm cotton ligatures were placed at intervals along the stump (Fig. 3). Considerable traction was necessary to move the tube after this; however, when no further fixation was used the tube did not stay in place. To accomplish this we made a loop in the tubing and snugly tied this with the still uncut ends of the arterial ligatures (Fig. 3). Experience in the proper tension to use while tying was necessary to achieve sufficient pressure without occluding the lumen of the tube. This was estimated by testing for free flow of blood in the tubing. A syringe with gentle suction may be necessary because of arterial spasm during the period of manipulation. Viscera and omentum were then replaced and the abdomen closed.

Heparin was used to decrease the likelihood of thrombosis about the tip of the tube, but heparin was not given until the 18th to 24th hour after operation because, in the few animals in which heparinization was begun before this time, serious hemorrhage occurred. Heparin was given with the perfusate in the range of 0.20 to 0.25 mg. per kilogram per hour. Most animals required 5 to 10 ml. of heparin solution per day which was added to the infusion flask. In animals infused with heparin and saline for several days there was no effect on the venous blood sugar or the islands of Langerhans. A modified Lee and White technic has been used to determine clotting time of venous blood, which was kept between 30 and 60 minutes. More heparin may be needed to maintain these clotting times if the infusion is continued more than a week. The chief complication in these experiments has been the tendency for thrombus formation in the region of the tip of the tube. In many instances this has occurred in spite of a prolonged clotting time. In addition to the clotting time there are two other factors which influence local thrombosis. One is the size of the artery and the rate of blood flow. The larger the artery the less likely is the occurrence of thrombosis. Another factor is the concentration of glucose perfused. Solutions ranging from 5 to 50 per cent have been tried but 5 to 17 per cent was found to be the useful range, since higher concentrations seem to favor the development of local thrombosis.

Post-operative care. All dogs were given 100,000 units of penicillin (intramuscularly) daily. The majority of the animals ate well on the first postoperative day and if no complications occurred, continued to eat well and to appear alert and well. Fever of approximately 1° C usually occurred on the second or third day but rarely thereafter.

The infusion tube was disconnected at its junction with the De Bakey tube once daily and observed for the return of blood in the tube under the arterial pressure. If blood failed to return it was almost always due to changes in the artery at the end of the tube and the experiment usually had to be terminated at that time.

RESULTS

A. *Physiological effects:* Because the infusion never included all of the pancreas, and because of the relatively small amounts of glucose administered, hyperglycemia of the peripheral blood did not occur. On the other hand, peripheral *hypo*glycemia was frequent. Many animals did not respond in this way but in them some surgical, technical or febrile complication was usually present. The rate of infusion of glucose was a critical

TABLE 1. ARTERIAL INFUSION OF GLUCOSE

Dog No.	Total period of infusion	Period of hypoglycemia*			Lesions of islands		
		Duration	Mean blood glucose	Average glucose infused	Hyperplasia	Degranulation	Hydropic
	Days	Days	Mg. per 100 ml.	Mg. per min.			
85	5	2	42 (3)†	55	0	?	0
70	12	3	36 (4)	120	+	0	0
56	18	4	33 (6)	260	+	+	0
77	9	6	33 (11)	84	0	0	0
78	17	8	46 (9)	84	0	+	0
75	12	9	26 (9)	42	+	+	+
82	10	9	35 (12)	42	+	+	+
85	14	13	27 (21)	81	+	+	0
60	16	15	26 (18)	100	+	+	+
83	19	18	37 (31)	83	+	+	0

* Defined as 50 mg. per 100 ml. or less.
† Numbers in parentheses indicate the number of determinations from which means were calculated. They were made during the periods of hypoglycemia which they define.

factor. Thus, in 8 animals infused with glucose in excess of 4.5 mg. per kilogram per minute the average blood sugar fell below 40 mg. per 100 ml. in all but one. When the rate of glucose infusion was less than 4.5 mg. per kilogram per minute, blood sugar values below 40 mg. per 100 ml. were found in only 2 of 8 animals. As a rule, the most successful and uncomplicated infusions of glucose were accompanied by a fairly well sustained hypoglycemia. Table 1 summarizes the information in 10 such animals which had hypoglycemia of 2 to 18 days' duration. The mean blood sugar level of the hypoglycemic period of each animal is given. As noted on the table, 50 mg. per 100 ml. was the highest value classified as hypoglycemia. A few of these animals had symptoms of hypoglycemia but such symptoms were infrequent. Figure 4 shows the longest sustained hypoglycemia of the series (dog 83). This animal had no symptoms of hypoglycemia which began in the presence of slight post-operative fever. Five per cent glucose was infused into the common hepatic artery until difficulty with the apparatus led to the terminal rise in blood sugar and necessitated the ending of the experiment. The dog was lively and well throughout this 20-day period.

On the contrary, dog 85 (Table 1), which had 13 days of hypoglycemia, died in hypoglycemic convulsions with a blood sugar of 10 while 5 per cent glucose was still being infused. Minor hypoglycemic reactions were suspected in a few other animals but the symptoms were quite indefinite.

In the literature, the experiments of a few hours' duration have shown the speed of response of the pancreas to glucose. Our more prolonged studies emphasize the capacity of the islands of Langerhans to respond to additional blood glucose in a powerful and sustained manner.

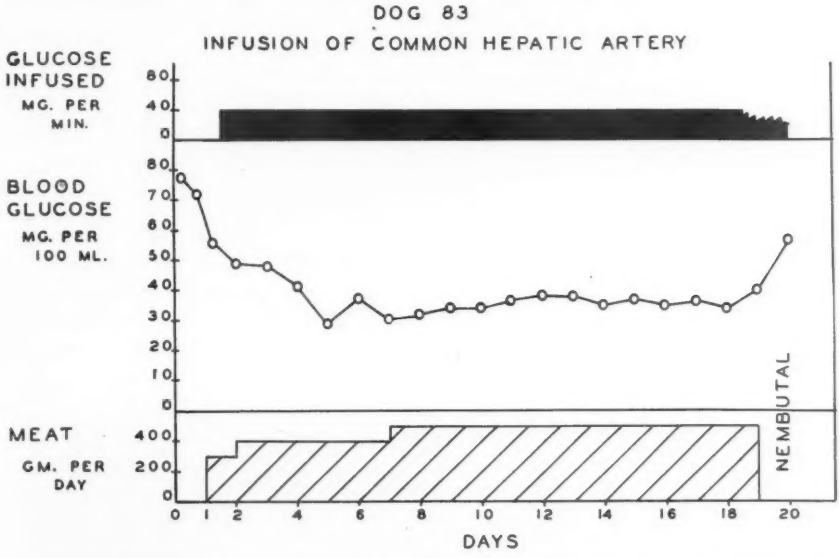

FIG. 4. *Dog 83: Infusion of Common Hepatic Artery with Glucose.* Hypoglycemia of peripheral venous blood was maintained for 19 days until trouble with the infusion apparatus occurred.

Since the common hepatic artery, which was most often used, sends blood to both the liver and the pancreas (Fig. 1) and since both of these organs are involved in the regulation of the blood sugar, additional experiments were performed to determine which of the two was principally responsible for the hypoglycemic effect. In 2 dogs, glucose was infused in the portal vein for 4 and 5 days. In both cases the peripheral blood glucose remained at normal levels. This is illustrated in Fig. 5 in which the normoglycemia of dog 89 is contrasted with the hypoglycemia of two dogs whose infusion included part of the pancreatic circulation.

B. *Anatomical effects on the islands of Langerhans:* Table 1 summarizes the anatomical findings. Five to 8 areas of the pancreas from head to tail were taken for section and stained with hematoxylin and eosin and by

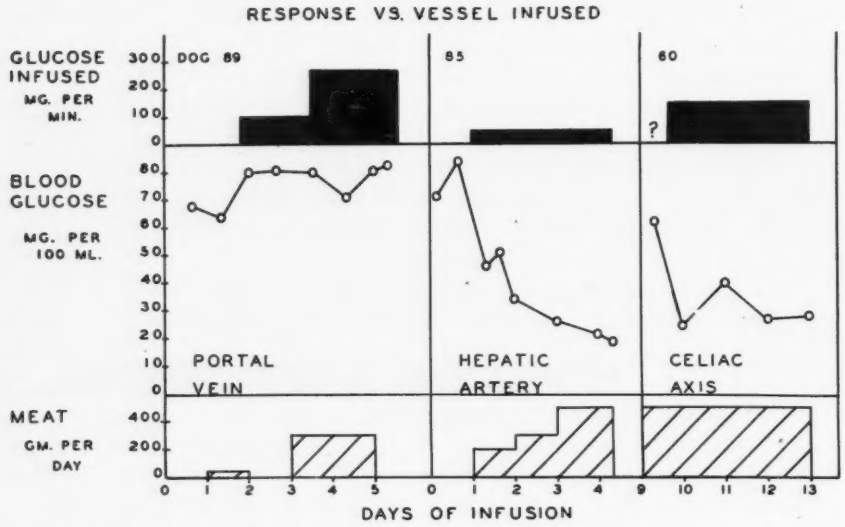

FIG. 5. *The response to the infusion of glucose into various blood vessels in dogs.* Glucose entered the pancreas through both arterial perfusions (cf Fig. 1.) but did not directly reach the pancreas through the portal vein.

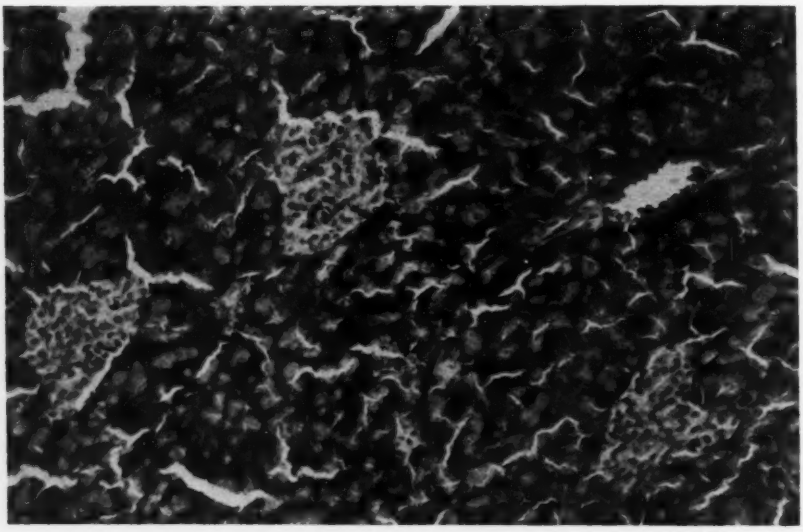

FIG. 6. Dog 56. Normal islands of Langerhans ×170.

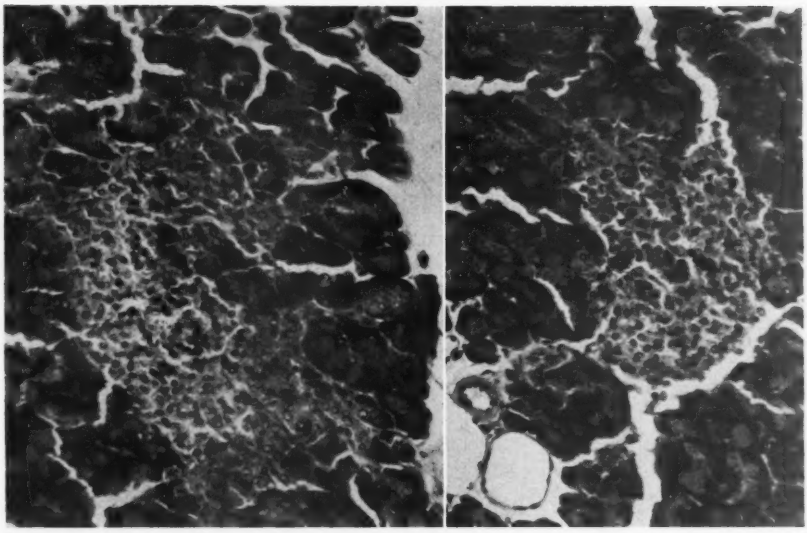

FIG. 7. Dog 56. Hyperplasia of islands of Langerhans (×170) from infused portion of pancreas. About 1 cm. of the print between these adjacent islands has been cut out to compose this figure.

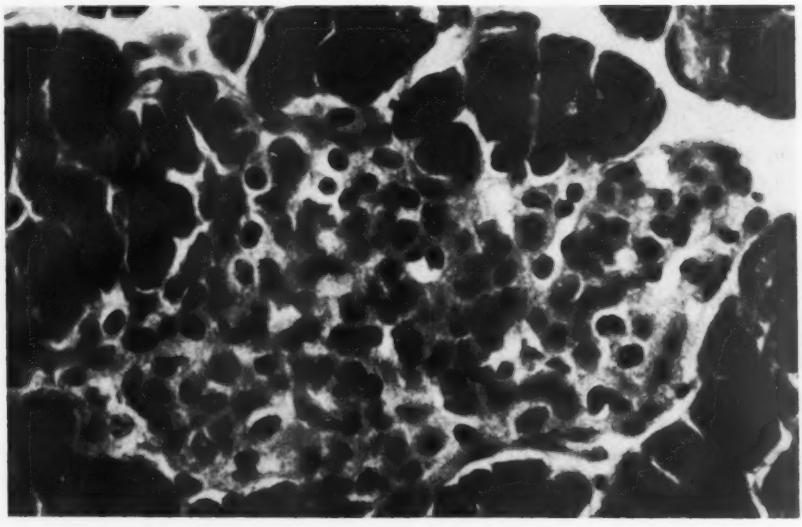

FIG. 8. Dog 60. Normal island of Langerhans ×600.

Gomori's method. It is important to emphasize the fact that all three types of change were focal and occurred only near the infused area. The large amount of normal tissue examined showed that these changes were not artefacts. As one would expect, inflammatory changes were frequently seen after such gross disturbance of the tissues. However, these were negligible in many experiments and damage of the islands was observed in areas essentially free of inflammation. Figure 7 illustrates hyperplasia of the islands and Figure 9 shows early hydropic degeneration, i.e. glycogen infiltration as demonstrated by Toreson (1950). Figures 6 and 8 show normal islands for comparison.

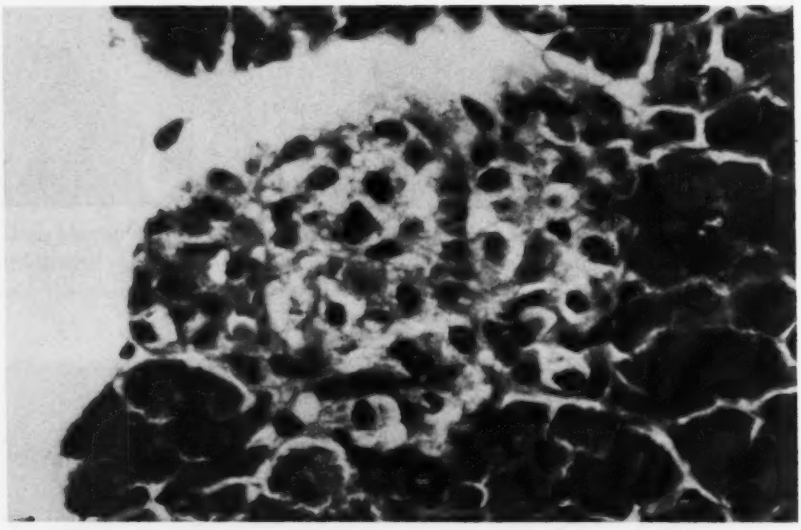

FIG. 9. Dog 60. Hydropic degeneration of island of Langerhans ×600.

The table indicates that hyperplasia and degranulation developed at times in the same pancreas. Occasionally these changes occurred in the same island, i.e. a large, hyperplastic island might contain degranulated beta cells. This combination of lesions has been observed by Wissler, Findley and Frazier (1949) in the rat.

DISCUSSION

An unsolved problem in these experiments has been the variable collateral circulation to the pancreas. Ideally, a known rate of perfusion through the organ under study would be desirable. The failure to achieve this presumably accounts for many irregularities in the results. Nevertheless, it is proper to discuss the conditions under which the three principal

findings, viz. peripheral hypoglycemia, localized hyperplasia and localized, early degenerative changes in the islands, have been observed. The occurrence of hyperplasia and of degenerative changes is not necessarily a contradiction if the local and variable nature of the hyperglycemia is recognized.

Peripheral hypoglycemia: Since hypoglycemia occurred only when the infusion included the pancreatic circulation it is assumed that this was caused by an increased secretion of insulin. Although many experimental and clinical observations have established this stimulating function of glucose, no such striking, prolonged demonstration of the effect of glucose has been made previously. The action of glucose on the secretion of insulin may depend not only on the concentration of glucose entering the pancreas but on the rate of blood flow. This is suggested by the development of degranulation of the islands at normal blood sugar levels in the experiments of Woerner (1938) and Vining (1949). An increased blood flow at a normal level of arterial blood sugar may well account for an increased secretion of insulin in those normal humans whose blood sugar fails to rise significantly after meals.

In Table 1, 10 dogs had a total of 132 infusion-days of which 87 or two-thirds were hypoglycemic. In each of the last 4 animals (82, 85, 60, 83) hypoglycemia was present on all but one day of the period of infusion. For this reason we shall assume that a high level of blood insulin was present in discussing the histological changes in these experiments.

Hyperplasia of the islands: The limited and localized occurrence of this response could hardly have been due to increased pancreatotrophic hormone (if there be such a hormone) for this ought to act upon all the islands. Hyperplasia of the islands has been seen in rats treated with glucose, even after hypophysectomy (Haist *et al.*, 1949). It has also occurred in guinea pigs given glucose intravenously with minimal or no hyperglycemia (Woerner, 1938). In our experiments hyperplasia occurred under different combinations of blood glucose and insulin reserve. These are among the factors which may account for the reported differences in the response of the islands of different species to glucose, pituitary extract and other influences. In addition, the tendency of insulin to suppress the growth of the islands (Haist *et al.*, 1949) is recalled. In contrast to their report, local hyperplasia has occurred in many of our animals despite the presence of a high level of blood insulin. In any case the effects upon the islands may be due to the particular equilibrium of such factors as hyperglycemia and the level of blood insulin.

Early degenerative changes: Degranulation and glycogen infiltration of the beta cells were observed only in areas near the infused artery. The fact that these lesions were best seen in regions free of inflammatory reaction

suggests that they were due to the infused glucose. The point to be emphasized is that this is the first time that these lesions have been reproduced in the presence of a high level of insulin secretion as indicated by the frequent finding of systemic hypoglycemia. Secretion of insulin presumably occurred during the development of these lesions in the experiments of Woerner (1938) and Vining (1949) in which the blood sugar remained normal or nearly so during the intravenous administration of glucose. On the other hand, many attempts to produce glycogen infiltration of the islands by the intravenous infusion of glucose have failed. In other situations insulin has prevented the development of such lesions (Best, *et al.*, 1942) or led to their reversal (Lukens and Dohan, 1942, Duff and Toreson, 1951). Here, as in the case of hyperplasia, the summation or equilibrium of several factors such as the level of blood insulin, hyperglycemia and perhaps other things may be more important than the variations observed in any one of them. These influences upon the islands of Langerhans may be summarized as follows:

Insulin: The protective and restorative action of insulin on the islands in experimental diabetes is recognized. In the recent study of Duff and Toreson (1951) the beneficial effect of insulin on glycogen infiltration (hydropic degeneration) of the islands was exerted in the presence of hyperglycemia. The work of Lukens and Dohan (1942) may well be in agreement with their observations. In the insulin treatment of cats made diabetic by previous pituitary extract, the few blood sugars (unpublished) determined in the first 2 to 3 days of treatment were all elevated. At the time the longer period of complete control of the diabetes and the striking physiological recovery obscured the importance of examining the early hours and days of treatment in detail. On the other hand, the protective influence of one or two normal blood sugars per day in the experiments on glucose-diabetes (Dohan and Lukens, 1948) compel us to be guarded in the interpretation of our few high blood sugars in the early days of insulin treatment. When all the reports on this subject are considered it seems clear that insulin *per se* is a factor of importance in the protection of the islands of Langerhans.

Hyperglycemia: When damaged islands are restored by reduction of diet or by treatment with phlorhizin (Lukens, Dohan and Wolcott, 1943), the initial change is not an increase in blood insulin (i.e. no insulin is administered), although an increased secretion of insulin must eventually occur as recovery progresses. The production of early degenerative changes by arterial infusion when the blood insulin is apparently elevated provides another situation in which the effects of hyperglycemia and the blood insulin level may be dissociated. It appears to us that the level of blood glucose is also a significant factor in the regulation and welfare of the islands,

at least in certain species. A supply of insulin and a normal blood sugar usually go together, and in the restoration of experimental lesions both may work together.

Other factors: These include toxins, of which alloxan is the experimental prototype, and hormones, of which the inhibition of the secretion of insulin by growth hormone (Anderson and Long, 1947) is an example. It would be an unwarranted digression to review all possible agents which may directly or indirectly affect the islands. However, it is essential to remember that hyperglycemia and the supply of insulin may not be the only factors involved.

In conclusion, arterial infusion of the pancreas with glucose has afforded a new situation by which to evaluate the response of the islands of Langerhans to blood insulin, hyperglycemia and other undefined factors.

SUMMARY

With the apparatus described, 5 to 17 per cent glucose has been continuously infused into the arterial circulation of part of the pancreas of normal dogs for periods as long as 18 days. Variations in blood flow were not controlled in these experiments. The results of this infusion of glucose were:

1. Peripheral venous hypoglycemia occurred for variable portions of the total infusion period. Peripheral hyperglycemia was not observed. Some of the experiments provided striking evidence of the power of glucose to stimulate the secretion of insulin as judged by the resulting hypoglycemia. Infusion of the portal vein with similar amounts of glucose did not alter the level of blood glucose.

2. Local hyperplasia of the islands of Langerhans was observed. In other areas degranulation and early glycogen infiltration (hydropic degeneration) were seen. Both types of local change developed when the blood insulin was elevated as indicated by the peripheral hypoglycemia. This differs from previous observations that insulin inhibits the growth of the islands and prevents or restores glycogen infiltration of the beta cells.

Taken in conjunction with previous work, the findings indicate that insulin, the level of blood sugar, and no doubt other factors influence the activity of the islands of Langerhans. The present results emphasize the need for considering the balance or equilibrium of such influences under various experimental conditions.

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INHIBITION OF THE CATABOLIC EFFECT OF ADRENOCORTICOTROPIC HORMONE (ACTH) IN RATS BY A DIET HIGH IN POTASSIUM CHLORIDE[1]

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INTRODUCTION

IT IS well recognized that hyperadrenocorticism whether spontaneous or induced by exogenous ACTH may be associated with a negative nitrogen balance and reduction of potassium concentrations in blood and tissues. To what extent these phenomena of nitrogen and electrolyte metabolism may be interrelated is not clear, although there are reports in the literature which suggest that an interrelationship is present. Pearson, Eliel, and Rawson (1950) have reported the metabolic balance data on a patient who was given extra KCl while receiving ACTH. These data show a slight reduction of questionable significance in the degree of negative nitrogen balance which coincides with the KCl administration. Kepler (1948) demonstrated that a patient with Cushing's syndrome who was in a negative nitrogen balance developed a positive nitrogen balance when given an additional 6 gm. of KCl per day. In view of these findings it seemed of interest to investigate the effect of a diet high in KCl on the nitrogen excretion of rats receiving ACTH. Evidence is presented in this paper which indicates that when rats are fed a diet high in KCl the increase in urinary nitrogen excretion produced by ACTH is inhibited.

METHODS

Four series of experiments were done. In each experiment "plateaued" female rats of the Long-Evans strain were used and there were two, three, or four groups of five animals in each experiment. Throughout each experiment the food intake for all animals was the same and was constant from day to day, 12 gm. of diet being eaten by each animal daily. Urines were collected daily. The methods for handling the animals, for maintaining a constant food intake, and for collecting the specimens are the same as those previously reported (Bennett and Li, 1949). Nitrogen was determined by the micro Kjeldahl procedure, and sodium and potassium were determined by use of the Perkin Elmer flame photometer, model 52A, using lithium as an internal standard. The authors are indebted to Dr. C. H. Li for generous supplies of ACTH which was prepared

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TABLE 1. EFFECT OF THE ACTH ON THE AVERAGE URINARY NITROGEN EXCRETION OF RATS FED A DIET HIGH IN KCl. NITROGEN EXPRESSED AS MG. PER 24 HRS.

	Pre-injection control period	ACTH treatment period									
		Urine N	Change‡	Urine N	Change‡	Urine N	Change‡	Average for total treatment period	Change‡	P_1	P_2
Daily dose of ACTH	0	6 mg.		6 mg.		9 mg.					
Series I High KCl	339 ± 5* (35)†	326 (15)	−13	350 (15)	+11	359 (10)	+33	344 ± 4	+ 5	0.10	
Series I Controls	348 ± 5 (35)	336 (15)	−12	374 (15)	+26	392 (10)	+44	364 ± 3	+16	<0.01	<0.01
Daily Dose of ACTH	0	5 mg.		10 mg.		15 mg.					
Series IV High KCl	331 ± 6 (45)	332 (10)	+ 1	328 (5)	− 3	329 (15)	− 2	330 ± 8 (30)	− 1	0.90	
Series IV Controls	319 ± 11 (45)	340 (10)	+21	348 (5)	+29	371 (13)	+52	357 ± 6	+30	<0.01	<0.01

* Standard deviation of the mean

$$\sigma = \sqrt{\frac{\Sigma d^2}{n(n-1)}}$$

† The number in parentheses indicates the number of individual observations.
p_1 Comparing the average of the total injection period with the pre-injection control period. p is from Fisher's table of t, where

$$t = \frac{M_1 - M_2}{\sqrt{\frac{\Sigma d_1^2 + \Sigma d_2^2}{(n_1 + n_2 - 2)} \times \left(\frac{1}{n^1} \quad \frac{1}{n^2}\right)}}$$

P_2 Comparing the total injection periods of the animals on the high KCl diet and the control diet.
‡ The difference between the injection period and the pre-injection control period.

by the method previously published (Li, Evans, and Simpson, 1943). The activity of these preparations were such that 0.2 mg. per day maintained the adrenal weight of 40-day old hypophysectomized rats.

RESULTS AND COMMENTS

Series I: In the first series, two groups of rats of five each were used. Each animal in the experimental group received a daily supplement of 600 mg. of KCl which was mixed with the diet; the other group served as the controls and received only the stock diet.[2] All rats were injected intraperitoneally with ACTH (Li 11623) for three days, receiving 6 mg. per rat per day in three equal doses. Since no effect on the nitrogen excretion was observed, the same amount of ACTH was given subcutaneously in six equally divided doses per day for the next three days. The dosage was then increased to 9 mg. per rat per day divided into six equal doses.

As may be seen in Table 1, 6 and 9 mg. of ACTH given subcutaneously caused a significant increase in nitrogen excretion in the control rats but not in the rats fed a high KCl diet. The difference in the average nitrogen excretion between the two groups during the period of ACTH injection was statistically significant, as a "p" value (Fisher, 1936) of less than 0.01 was found when the average nitrogen excretion for the two groups during the injection period was compared. The body weight data show that while the rats on the high KCl diet did not lose weight, the animals receiving ACTH without the added KCl did. The animals fed the high KCl diet actually showed a slight increase in body weight.

Series II: In the second experiment ten rats were divided into two groups of five animals each. Following a 9-day control period, each animal in the experimental group was given an additional 600 mg. of KCl per day for the next seven days to determine whether a high KCl diet alone would change the urinary nitrogen excretion. The data from this experiment are presented in Fig. 1, and show clearly that when ACTH is not administered the addition of KCl to the diet does not alter the nitrogen excretion. On the sixteenth day each rat was given 9 mg. of ACTH (Li 11624), subcutaneously in four equal doses per day. Since this amount of ACTH had no effect on the nitrogen excretion of either group of rats, the amount of ACTH given was increased to 17.5 mg. per day. At 17.5 mg. per day, the animals eating the diet without the extra KCl showed an increase in urinary nitrogen as compared to the pre-injection control period and as compared to the rats on the high KCl diet. Statistical analysis gave "p" values

[2] The diet used consisted of ground whole wheat 67.5%, casein 15.0%, whole milk powder, 10.0%, NaCl 0.75%, $CaCO_3$ 1.5%, and hydrogenated vegetable oil 5.25%. To each kg. of diet were added 3.5 gm. of fish oil concentrate containing 3000 USP units of vitamin A and 400 chick units of vitamin D per gm.

of less than .01 for each comparison. The group on the high potassium diet showed a slight increase in urinary nitrogen during ACTH administration, but this was not statistically significant. There was no consistent change in the potassium or sodium excretion during the ACTH injection period. Fecal analyses were also done in this series and showed that neither the diet high in KCl nor the administration of ACTH altered the absorption of nitrogen from the gastrointestinal tract.

Series III: Since sodium and potassium often have an inverse relationship in biological systems, this experiment was done to determine whether a high NaCl diet had any effect on the action of ACTH on nitrogen excre-

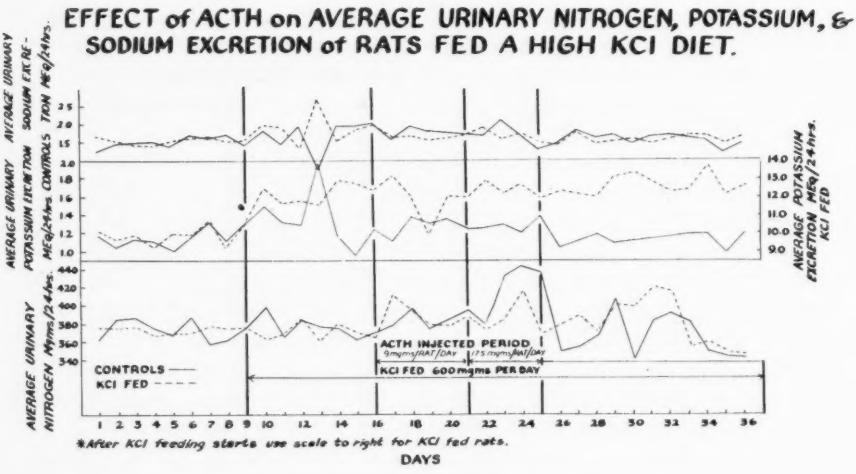
EFFECT of ACTH on AVERAGE URINARY NITROGEN, POTASSIUM, & SODIUM EXCRETION of RATS FED A HIGH KCl DIET.
AVERAGE URINARY POTASSIUM EXCRETION FROM CONTROLS (MEq/24 hrs.)
AVERAGE URINARY SODIUM EXCRETION (MEq/24 hrs.)
AVERAGE URINARY NITROGEN (Mgms./24 hrs.)
AVERAGE POTASSIUM EXCRETION (MEq/24 hrs.) KCl FED
ACTH INJECTED PERIOD
9 mgms/RAT/DAY
15 mgms/RAT/DAY
20 mgms/RAT/DAY
CONTROLS
KCl FED
KCl FED 600 mgms PER DAY
*After KCl feeding starts use scale to right for KCl fed rats.
DAYS

FIG. 1

tion. In this experiment three groups of five rats each were used. One group served as controls; one group received 600 mg. of additional KCl per rat per day, and one group received 600 mg. of additional NaCl per rat per day. Following a 7-day control period, ACTH (Li 11623) was injected in doses of 9 mg. for two days. 15 mg. for 2 days, and 20 mg. for 3 days; in each case the injection was given subcutaneously in four equal doses. Only a transient rise in nitrogen excretion was produced in the controls. Because of this minimal effect, 10 mg. per day per rat of another lot of ACTH (Li 11624) was given subcutaneously. As may be seen in Fig. 2 the rats on the control diet as well as those on the high NaCl diet showed an increase in urinary nitrogen while receiving ACTH. The rats on the high KCl diet did not show an increase in urinary nitrogen. Again statistical analysis showed that the nitrogen excretion of the high KCl group during the last

two ACTH treatment periods was significantly lower than that of either of the other two groups.

Series IV: The fourth set of experiments was undertaken to determine whether the high potassium diet was inhibiting the action of ACTH on the adrenal gland. Four groups of five rats each were used. Two groups received 600 mg. extra KCl per rat per day and two groups served as the controls. One control group and one high KCl group received ACTH (Li 11666) in increasing doses (See Table 1) until an increase in urinary nitrogen was observed in the control animals. After the last day of injection all rats were sacrificed by ether, the left adrenal of each rat excised, dissected free of fat and fixed in 10 per cent neutral formol. Two days later

TABLE 2. EFFECT OF ACTH ON THE ADRENAL WEIGHT OF RATS FED A DIET HIGH IN KCl

		Average weight of adrenal gland mg.
High KCl Diet plus ACTH	(5)†	67 (49–86)*
High KCl Diet without ACTH	(5)	41 (28–51)
Control Diet without ACTH	(4)	39 (32–45)
Control Diet plus ACTH	(5)	63 (57–91)

* Range of weight.
† Number of animals in the group.

the adrenals were weighed. The average weights are presented in Table 2. The data presented in the table show that there was no difference in the adrenal weights of the rats receiving ACTH whether or not they were fed extra KCl. Frozen sections were prepared and stained for lipid using Red O. Although the cortices of the adrenals of the rats injected with ACTH were increased in size as compared with those of the control rats, there was no difference in the lipid distribution whether the animals were on the control diet or the diet high in KCl.

The metabolic data on the control group and on the high KCl-fed group which received ACTH are presented in Table 1. The nitrogen data are consistent with those obtained in the first three experiments. That is, there was a significant increase in the urinary nitrogen of the control animals when they received ACTH, but there was no increase in the urinary nitrogen of the animals on the high KCl diet during ACTH treatment.

From the evidence presented above, it seems clear that a high KCl diet inhibits the increase in nitrogen excretion produced by ACTH in normal female rats of the Long-Evans strain. It would appear clear that the potassium does not inhibit the stimulation of the adrenal by ACTH, since the adrenal weights of the rats receiving ACTH in both the high KCl group and the group on the control diet were the same and the adrenals of the

two groups were indistinguishable histologically. The inference from this is that a high KCl diet must in some way inhibit the peripheral action of the "11-17 oxysteroids." It must be stressed that in these experiments we were using essentially minimum effective doses of ACTH. In each case the initial dose of ACTH was too small to increase the urinary nitrogen and

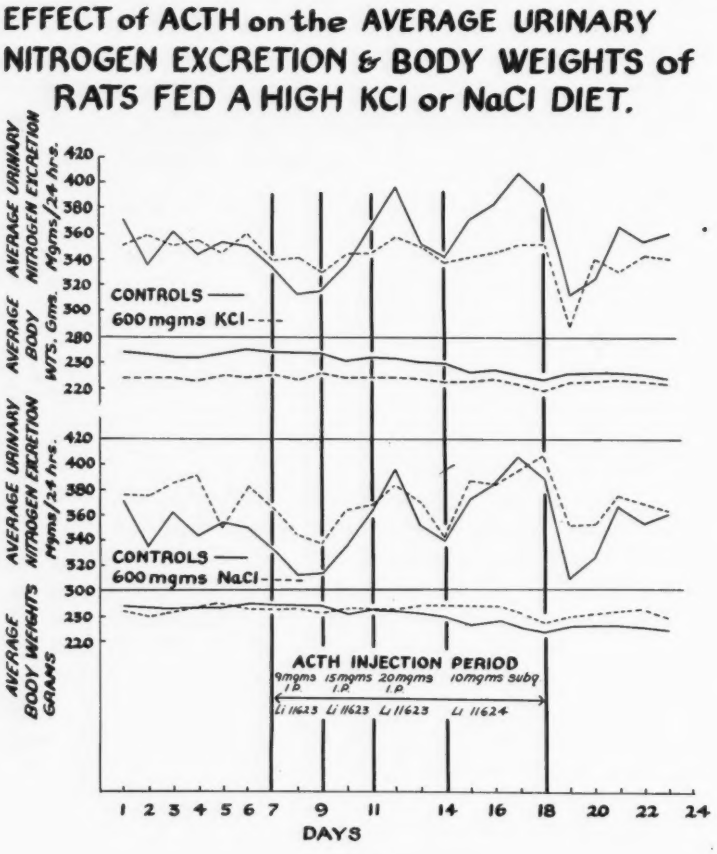

FIG. 2

the dose was then stepped up until an effect was observed in the control animals. Probably if sufficiently large doses of ACTH had been employed an increase in urinary nitrogen would have been found in the animals fed the diet high in KCl. The observed slight increase in the high KCl animals in Series I and II are in accord with this supposition. At the moment no facts are at hand regarding quantitative relations between the amount of

KCl fed, the dosage of ACTH and the inhibition of this effect on ACTH on nitrogen metabolism.

SUMMARY

Four series of metabolic experiments were carried out in which groups of 5 normal "plateaued" female rats were maintained on the stock diet, on a diet high in KCl, or on a diet high in NaCl. The food intake was constant and urines were collected daily for total nitrogen determination. All animals were given ACTH in increasingly large doses until a significant increase in urinary nitrogen was observed in the animals on the control diet. A similar increase in urinary nitrogen was observed in the animals on the high NaCl diet. No increase in urinary nitrogen was observed in the animals in the high KCl diet. The adrenal weight and histology of the animals receiving ACTH were indistinguishable whether they were on the control diet or on the high KCl diet. It is concluded that the increase in urinary nitrogen produced by ACTH may be inhibited by a diet high in KCl under the conditions of these experiments and that, judged by the criteria of adrenal weight and histology, the high KCl diet does not inhibit stimulation of the adrenal cortex by ACTH.

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ADRENAL CORTICAL INSUFFICIENCY IN THE RHESUS MONKEY[1]

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THE state of total adrenal cortical insufficiency, which inevitably has a fatal termination in the absence of therapeutic measures, has been well characterized in respect to changes in chemical composition of the body fluids and tissues and in attendant decline in body weight and muscular strength. Obviously, morbid processes are at work but their specific nature has not been determined. It would be of great interest to know, for instance, whether there is a stepwise disruption of physiological mechanisms and to which of these can death be attributed. An understanding of any part of the adrenal deficiency picture would in some degree contribute to the larger problem of the peripheral effect of the adrenal cortical hormones and perhaps provide implications as to means of correcting the deficiency.

The continued cataloguing of facts relating to the adrenal deficiency state will contribute to its eventual elucidation but there is particular need at this time for intensive study of single species throughout the period of survival, thus giving the continuity to the data that makes comparisons valid and allows analyses and interpretations of the many coexistent changes to be made. An excellent recent study of this type has been made on the goat by Cowie and Sewart (1949). We chose to make a similar study on the rhesus monkey because of: 1) the ease with which he can be maintained under standardized conditions; 2) the feasibility of making continued venipunctures, and for the added reason 3) his many points of similarity with man lend a special meaning to any observations made upon him. Only scattered observations on adrenal insufficiency in various species of monkeys have been recorded (Kahn, 1912; Firor and Grollman, 1933; Britton, Kline and Silvette, 1938; Brittin and Silvette, 1936; and Hartman and Brownell, 1949).

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Observations of the animals in respect to body weight, activity, appetite, and measurements of the pertinent chemical constituents of the blood were made for a period before adrenalectomy and followed continually thereafter until death.

MATERIALS AND METHODS

Animals

Male monkeys (*Macaca mulatta*) of unknown age but assumed to be juvenile on the basis of dentition, body weight (4–6 kg.) and sexual development were employed in this study. They were quartered in individual cages located in an air-conditioned room maintained at a constant temperature of from 78° to 80° F. Food[4] and water were supplied *ad libitum* throughout the experiment.

Prior to operation, blood samples were withdrawn at 3-day intervals to establish control values for the various blood constituents to be determined. The samples were removed from the superficial veins on the posterior aspect of the leg during the control and initial experimental periods, but as adrenal insufficiency became manifest, the leg veins gradually collapsed, making it necessary to resort to femoral and occasionally to cardiac punctures to obtain the required volume of blood (5 ml.). The blood was withdrawn under oil to minimize hemolysis; 2 ml. were immediately delivered into a heparinized vial for the determination of whole blood constituents and hematocrit, and the remainder was allowed to clot in a centrifuge tube for serum electrolyte analyses. Postoperatively, the animals were bled at 3-day intervals, a final sample being taken as near death as possible. The initial bleedings after adrenalectomy were scheduled to permit observations, on at least one animal, for each of the first three postoperative days.

Operative procedures: Single-stage, bilateral adrenalectomy was performed in the rhesus monkey in the following manner: The animals were anesthetized by intravenously administered pentobarbital sodium (27 mg./kg. body weight), and appropriately positioned for a dorso-lateral incision, with a small rolled sheet placed to elevate and flex their midsection. Strict aseptic technique was used throughout.

The incision was made along the caudal border of the 12th rib, extending from a point 1–2 cm. lateral to the spinous processes to the posterior axillary line. When the superficial muscles had been divided down to the plane of the thoracic cage, they were retracted cephalo-medially in order to expose the 12th rib, which was then resected sub-periosteally (on the right side only). Tension on the incision was released and the deeper muscle groups divided along the caudal border of the 12th rib bed to the level of the transversalis facia. The retroperitoneal space was entered at the lateral border of the quadratus lumborum, the peritoneum being carefully dissected, and the transversalis fascia divided along the length of the incision.

In the retroperitoneal space, the upper pole of the kidney was palpated by an exploring index finger and, cephalo-medial to it, the adrenal was located. Using a small pledget of gauze held in the tip of an Overholt right angle hemostat as a blunt dissector, the tissues surrounding the gland were gently swept away until it could be visualized and

[4] Vitaminized casein 15%, vitaminized corn oil 2% (Shaw and Greep, 1949), salts 3% (Shaw, 1947), yeast 3%, sucrose 63%, whole liver substance 2% (The Wilson Laboratories, Chicago), Cellu flour 13% (Chicago Dietetic Supply House, Chicago), and ascorbic acid 0.1%. This ration has been used in this laboratory for a number of years and has been found to be most satisfactory for growth.

freed from contiguous structures, except for a pedicle composed of a few connective tissue strands and the veins supplying the gland. This pedicle was grasped with a right angle hemostat, the gland amputated and the pedicle doubly ligated. The wound was closed in layers, using interrupted cotton technique. Both glands were removed in 2½–3 hours. For prophylactic purposes, 300,000–600,000 units of penicillin were given immediately after operation. The animal received no supportive therapy.

The main surgical difficulties in this operation are the following: 1) pneumothorax may result from perforation either of the pleura during resection of the 12th rib, or of the diaphragm where it crosses the median extremity of the incision; and 2) blood loss—on the right side, the gland is closely applied to the *vena cava*. The hemorrhage resulting from inadvertent entrance into this vessel can be fatal. Death was averted on one such occasion in our experience by the prompt transfusion of whole blood. The need for careful dissection in this region can not be overemphasized. The opening of smaller veins in a poorly exposed field where they can not be located easily will cause persistent, irksome bleeding, but this is usually not dangerous. The clamping of these small vessels with McKenzie silver clips in regions of limited maneuverability has proven advantageous. The whole surgical procedure, when properly executed, entails but negligible blood loss.

We have experienced a small but significant number of unexplained deaths within the first 48 hours after surgery. The available evidence implicates respiratory failure.

Analytical methods

The serum was analyzed for sodium and potassium by an internally compensated Perkin-Elmer flame photometer, and for chloride by the mercurometric method of Schales and Schales (1940).

Inorganic phosphorus was determined by the method of Fiske and Subbarow (1925), 0.1 ml. of whole blood being employed for each analysis. One milliliter of the remaining heparinized sample was deproteinized by Haden's (1923) modification of the Folin and Wu method and the resulting protein-free filtrate analyzed for the following constituents: glucose, by the method of Nelson (1944), amino acid nitrogen by the method of Frame, Russel and Wilhelmi (1943), and nonprotein nitrogen by the method of Koch and McMeekin (1924). All these procedures had to be modified somewhat to permit the utilization of smaller volumes of protein-free filtrate, i.e., 0.2 ml. for glucose and 1 ml. for amino and nonprotein nitrogen determinations. All photometric procedures were carried out with the Evelyn photoelectric colorimeter.

RESULTS

I. *Recovery from trauma of operation*

The animals were generally adrenalectomized in the afternoon, and on the following morning it was difficult to discern any effects of the operation either on their general appearance or their behavior. They were aggressive when approached and moved about in their cages perhaps only slightly less agilely than usual.

II. *Visible changes in the animals during the postoperative period*

The commonly-reported finding of weight loss following adrenalectomy was consistently seen in these monkeys. This loss, plotted as a percentage deviation from the preoperative weight for the 4 monkeys studied most in-

tensively, is shown in Figures 1 and 2; their average weight loss was 1.1% of the preoperative weight per day.

Less tangible but as striking were the changes in the general appearance and behavior of the animals following adrenalectomy. As adrenal insufficiency progressed, the animals became less aggressive and more tractable when approached and handled. A decrease in the general muscle tone could be felt. They were more inclined to sit quietly in the bottom of their cages, hunched over, rather than being alertly poised on their perches, as was their usual custom. Their faces took on a drawn, pinched expression and

FIG. 1. Animal in severe adrenal insufficiency showing characteristic hunched posture and pilo-erection.

their muzzles, normally tannish in color, took on a grayish hue. Pilo-erection, as shown in Figure 3, was a consistent finding throughout the postoperative period. These changes in general appearance following adrenalectomy for one of the monkeys can be seen in Figure 4, and the behavior changes can be inferred from this series of photographs.

We also observed, in a rough fashion, another of the commonly reported findings of adrenal insufficiency—anorexia. Due to wastage, food and water consumption is hard to measure in monkeys. The decrease in food consumption following adrenalectomy, however, was so gross as to be readily evident. Even in the advanced stages of adrenal insufficiency the monkey

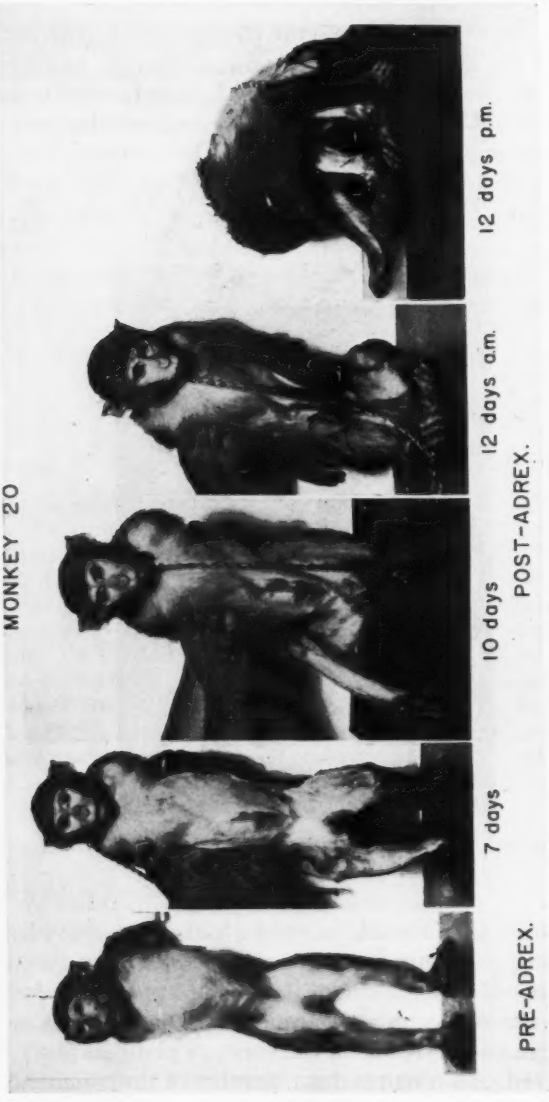

FIG. 2. Photographic record of the alterations in general appearance of a rhesus monkey following adrenalectomy.

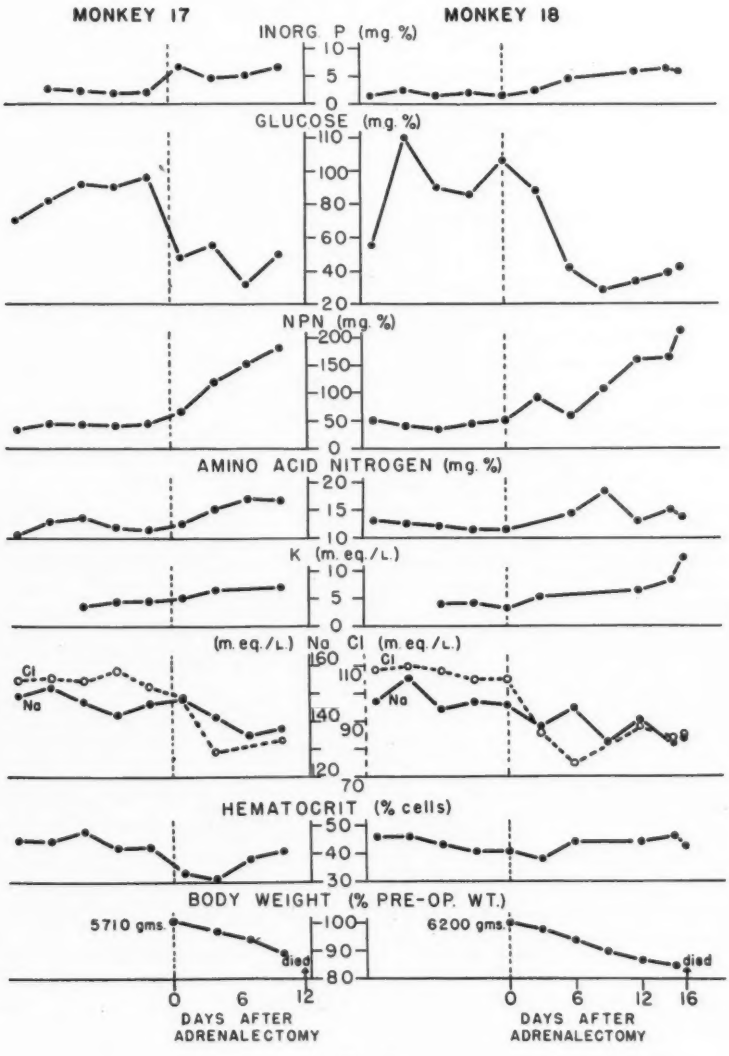

FIG. 3

FIG. 3 and 4. Changes wrought in the levels of certain blood constituents by adrenalectomy in the rhesus monkey.

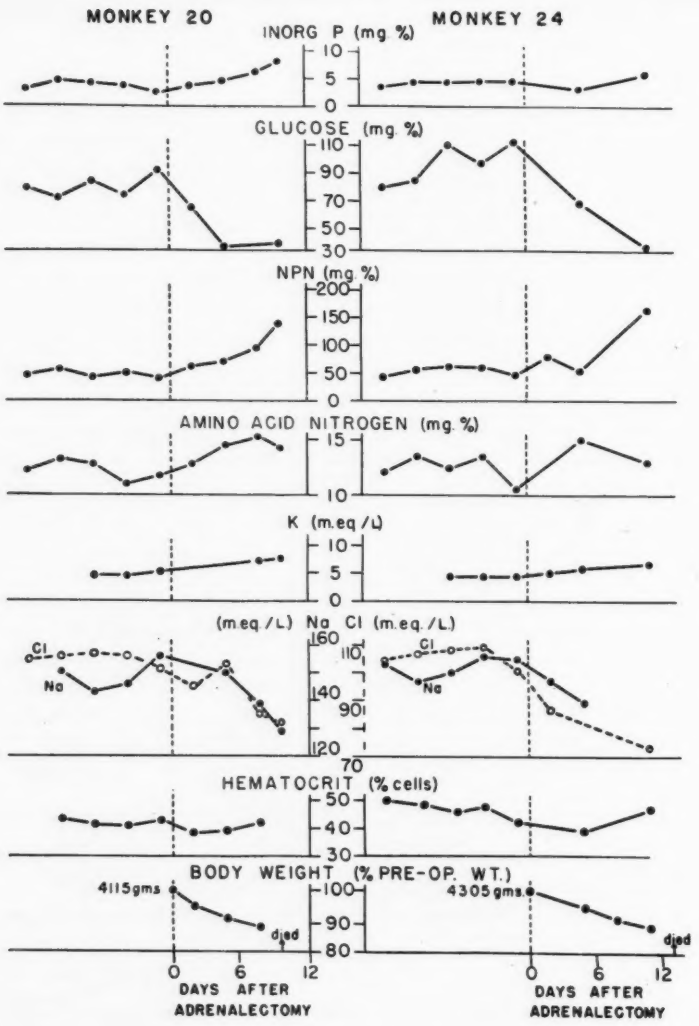

FIG. 4

will avidly snatch at a choice morsel such as a banana, but, once having obtained the food, will eat little or none of it.

III. *Duration of survival following adrenalectomy*

For the 4 monkeys reported in detail in this study and as a consequence bled every 3 days, the average survival time was 13 days, the extremes be-

ing 10 and 16. Three other monkeys, bled irregularly or not at all in the postoperative period, had an average survival time of 19 days, with a range of 18 to 20.

Adrenalectomized animals are well known to have a decreased resistance to infectious processes. No evidence was found during the observation period or at necropsy that such processes had contributed to the death of these monkeys. In contrast to the findings in other species, diarrhea was observed only infrequently.

Because of the operation of these aforementioned factors and the small number of animals studied, no serious attempt was made to correlate size of animal and length of survival, although a direct relationship between them was suggested by the data.

IV. *Changes in blood constituents following adrenalectomy*

The average values for several blood constituents obtained from intact monkeys are shown in Table 1. The changes in these substances following

TABLE 1. CONTROL VALUES—BLOOD CONSTITUENTS IN THE NORMAL RHESUS MONKEY

Constituent	Source	No. animals	No. observations	Average value
Non-protein nitrogen (mg. %)	Whole blood	9	36	45.5 (±1.02)*
Inorganic phosphorous (mg. %)	Whole blood	9	33	3.44 (±0.21)
Blood glucose (mg. %)	Whole blood	8	31	93.1 (±3.16)
Amino acid nitrogen (mg. %)	Whole blood	9	34	12.8 (±0.28)
Sodium (meq./L.)	Serum	10	24	148.5 (±0.99)
Potassium (meq./L.)	Serum	10	23	4.53 (±0.08)
Chloride (meq./L.)	Serum	9	31	105.7 (±0.61)
Hematocrit (% cells)	Whole blood	9	42	44.4 (±0.28)

* Standard error of mean.

adrenalectomy are shown graphically in Figures 1 and 2, and are summarized below:

Inorganic phosphorus—rises steadily following adrenalectomy, the highest value being recorded just before death.

Glucose—drops precipitously following adrenalectomy, the minimal values being reached near the middle of the survival period.

Non-protein nitrogen—starts rising after adrenalectomy, the extent of the increase apparently is dependent upon the length of the survival period.

Amino acid nitrogen—rises postoperatively, achieves a maximum near the middle of the survival period and tends to return to normal before death.

Serum potassium—rises inexorably from the time of operation until death.

Serum sodium—falls in an erratic fashion, tending to show slight increases on occasion, although the trend is unmistakably downward.

Serum chloride—pursues much the same course as does serum sodium.

Hematocrit—falls sharply following adrenalectomy, then tends to rise, being at or near the normal value prior to death.

V. *Post-mortem findings*

Upon autopsy, no macroscopic pathology was visible in any of the major organs. In contrast to the findings in other species, notably the dog, the gastro-intestinal tract appeared normal. Despite the fasted state of the animals prior to death, the gut contained formed fecal matter, brownish-green in color, but no evidence of hemorrhage was detected.

DISCUSSION

The decline and eventual death of an animal following extirpation of the adrenals illustrates in an inimitable fashion the utter dependence of the organism on these glands for life. Observation of these animals gives the impression of body-wide disruption of function, and this veritable plethora of disturbances impedes rather than assists an elucidation of adrenal cortical physiology. Specific information as to the cause of death from adrenal insufficiency is not readily evident from an examination of the changes in blood constituents. It is quite possible that such information is not to be found in the altered blood picture alone; from our observations it seems more likely that the summated effects of several coexistent abnormalities, such as severe inanition and hemodynamic upsets, for example, may prove fatal.

The length of the survival period following adrenalectomy is probably the resultant of many factors. It is quite likely that the survival times of our animals were decreased by periodic bleedings, as is indicated by the discrepancy in average survival times between the periodically bled and the irregularly or non-bled monkeys mentioned above. On several occasions, convulsions were observed which were taken to be of hypoglycemic origin. They occurred only after a prolonged period of restraint attendant upon the difficulty of bleeding an animal in advanced adrenal insufficiency. We feel the traumata of these occasions also served to decrease the length of the survival period.

The distinctive pattern of amino acid nitrogen concentrations following adrenalectomy is of interest since it was observed in every animal studied and, to our knowledge, has not been previously reported. The initial rise in amino acid nitrogen concentration followed by a decline approaching the normal level as adrenal insufficiency progressed can not be readily interpreted in the light of experimental evidence at hand and must await fur-

ther experimentation for its elucidation. Contrary to the present findings, Friedberg and Greenberg (1947) have reported a decrease in the plasma levels of amino acid nitrogen in the adrenalectomized, fasting rat.

The dramatic and often reported rise in blood non-protein nitrogen (NPN) following adrenalectomy was also evident in the rhesus monkey with up to a fivefold increase being attained in this species. As in other species (Noble, 1950), the highest NPN value was observed in the animal which survived the longest following the operation (monkey 18). Although the rise in blood NPN concentration during adrenal insufficiency has been generally ascribed to an impairment of renal function, it seems uncertain whether the two- to threefold increase in blood inorganic phosphorus levels can be accounted for in like manner. It is possible that the severe inanition, the profound disturbance in carbohydrate metabolism and the consequent tissue breakdown as evidenced by marked weight loss, may be contributory in this regard.

As in the New World monkey (Britton and Silvette, 1936), adrenalectomy in the rhesus monkey was followed by a rapid fall in blood glucose which reached a minimal level (c. 30 mg. %) near the middle of the survival period. Since the animals refused food almost immediately after the operation, the hypoglycemia could be most readily explained in terms of the fasting process. In his review, Noble (1950) states that "it is now apparent that there is a certain degree of species difference, and the time of onset of hypoglycemia is directly related to the duration of fasting." This may be an oversimplification of the problem since we have returned the blood sugar of two adrenalectomized monkeys to the normal level by the administration of cortisone despite continued anorexia (unpublished data). Although inanition is probably involved, an impairment of gluconeogenic processes may have played a major role in the hypoglycemia observed in the present study.

The changes in the serum electrolytes following adrenalectomy in the rhesus monkey do not differ markedly from the findings in other species (following this operation). We observed a fall in serum sodium (to levels approximating 90% of the control values) and chloride, and a rise in serum potassium. The smaller number of observations on serum potassium reflects the difficulty of obtaining unhemolyzed blood samples from animals which are difficult to bleed. Three points of especial interest can be noted in the data, however: first, the decrease in serum chloride values is equal to or greater than the decrease in serum sodium values. Especially in monkeys 17 and 24 can it be noted that, on a milli-equivalent for milli-equivalent basis, appreciably more chloride is lost from the blood than is sodium. This is in contrast to the dog where the ratio of serum sodium loss to serum chloride loss is roughly 1.6:1 (Muntwyler *et al.*, 1940) or in the rat where

the same ratio is almost 3:1 (Buell and Turner, 1941), although Harrison and Darrow (1938) report a 1:1 ratio. Britton, Kline and Silvette (1938) found the ratio of serum sodium to serum chloride loss to be 1.7:1 in the white-faced monkey. Employing average control values as a point of departure and contrasting them with the last observed values for serum sodium and chloride, the ratio of milli-equivalents of sodium lost from the serum to that of chloride lost is 0.6:1 in this study. Why this ratio should obtain in the case of the adrenally insufficient rhesus monkey can be, at present, only a matter for conjecture. Perhaps the production of fixed acid in an animal eating poorly or not at all could account for the displacement of chloride from the blood. Secondly, it can be seen that the decreases in serum sodium and chloride values pursue an erratic course when plotted as a function of time, although the overall trend is unmistakably a downward one. This is especially evident in the case of monkey 18. We feel reasonably confident that these deviations from the predominant pattern do not reflect analytical artifacts. Again, it is only possible to speculate as to their causation. These alterations in serum sodium and chloride concentrations show no correlation with changes in hematocrit, minimizing any influence of variations in plasma volume on these values. The temporary increases might be a reflection of the irregular eating habits of the animals, food consumption acting to elevate these values temporarily. It is noteworthy that these fluctuations are not observed in the serum potassium values; their climb after adrenalectomy is a steady and unbroken one. Thirdly, that while the electolyte values become progressively more unphysiological, the greatest part of the change occurs roughly within the first third of the survival period. Thus, for two-thirds of the postoperative period, the animals are living with markedly deranged serum electrolyte concentrations. This finding tends to minimize the importance of altered serum electrolyte values as a contributory factor in the death of adrenally insufficient animals.

The changes in hematocrit following adrenalectomy are rather unusual. Hemoconcentration in adrenal insufficiency is a commonly reported finding; yet, the first change in hematocrit following adrenalectomy in the rhesus monkey is in the direction of hemodilution. This is followed by an upward trend resulting in hematocrit values at or near to normal just before death. It will be noted that the prevalent trend in the control hematocrit values is a downward one, and a possible explanation for the observed postoperative hematocrit values may be found if this trend is kept in mind. The animals were bled every 3 days, both pre- and postoperatively, 5 cc. being withdrawn at each bleeding. This rate of blood loss may have exceeded the capacity of the hematopoietic systems to replace the formed elements in the blood; hence, the observed steady drop in hematocrit

values during the control period. This downward course was continued into the early part of the post-adrenalectomy period. As adrenal insufficiency became more pronounced, this trend may have been offset to some extent by a steadily increasing hemoconcentration. Just before death, the algebraic summation of these two antagonistic influences gave hematorcrit values close to those recorded for the first bleedings in the control period.

SUMMARY

Single-stage, bilateral adrenalectomy was performed upon male, rhesus monkeys. The animals were bled every 3 days in the pre- and postoperative periods for the measurement of certain blood constituents. The animals were also photographed at intervals throughout the duration of the experiment.

The average survival time for the 4 monkeys reported in detail in this study was 13 days. A steady weight loss postoperatively was evident in all of them. Decrease of motor activity and progressive enfeeblement were also observed in all of the animals.

Blood NPN and inorganic phosphorus rose after adrenalectomy as did serum potassium values. Blood glucose dropped and serum sodium and chloride values dropped also, although their downward course was an erratic one in several cases. Blood amino acid nitrogen rose following adrenalectomy, achieved a peak around the middle of the postoperative period, but tended to subside before death occurred. Hematocrit fell sharply immediately following operation but tended to return to normal before death.

The influence of certain factors obtaining in the experimental situation upon survival is discussed in some detail.

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SALINE HYDRATION AND DIURESIS AFTER ADRENALECTOMY*

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ANIMALS in adrenal insufficiency, when hydrated orally with water, cannot excrete the water at a normal rate. A delay in excretion is also obtained when the water is administered as glucose or 0.85% saline solution (Gaunt, 1944; Hays and Mathieson, 1945; Reforzo-Membrives, Power and Kepler, 1945). Even the administration of desoxycorticosterone acetate (DCA) or adrenal cortical extracts will only maintain a normal response to hydration for a short period of time (Gaunt, 1944; Gaunt, Liling and Cordsen, 1945; Hays and Mathieson, 1945), although injections of epinephrine will increase the diuresis. The adrenalectomized animal, receiving excess fluids, will also easily develop the syndrome of water intoxication. Adrenal steroids, particularly DCA, will offer good protection against water intoxication, without, however, markedly increasing urine output. Various theories have been elaborated in attempts to explain the failure of adrenalectomized animals to excrete water, glucose solution or 0.85% saline at a normal rate.

It is known that the adrenalectomized animal loses sodium chloride and recent studies have shown that depletion of sodium chloride in the extracellular fluid of normal dogs and rats can interfere with urinary excretion (Bristol, 1951). Inasmuch as sodium alone can be an important factor in urine excretion, the subject of diuresis in adrenalectomized animals was reinvestigated. In the present study, adrenalectomized animals were hydrated not only with water and isotonic saline but with various intermediate concentrations of sodium chloride. It was found, depending on the actual concentration of salt administered, that the untreated adrenalectomized animal may excrete urine at a normal rate.

METHODS

Male rats weighing between 175 to 225 grams were used. They received a normal stock diet with water *ad libitum*. The animals were hydrated according to the method of Hays and Mathieson (1945), in which four doses of the test solution are administered at hourly intervals by stomach tube. Urine volume, timed from the first hydration, is measured each hour for a period of seven hours. The hydration tests were carried out

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16 to 18 hours and 7 days after adrenalectomy. The rats were hydrated in groups of two in metabolism cages and urine excretion is calculated in all figures and tables as the per cent of the hydration doses excreted. Excretion studies were made at the same time on normal animals fed the same stock diet and housed under the same conditions. Urine chlorides were determined on the total urine collected during the 7 hour period.

RESULTS

Normal animals: Normal control animals excreted hydration doses of water quite rapidly and by the 7th hour between 70 to 80% of the total administered water was eliminated (Fig. 1). This excretion changes little

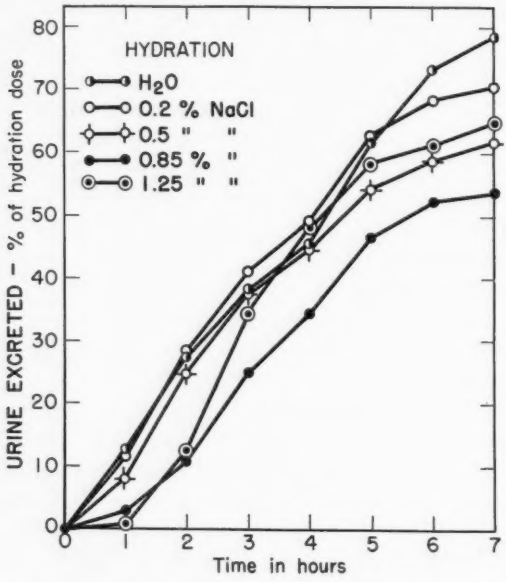

FIG. 1. Average urine excretion of normal rats hydrated with water and various concentrations of saline.

when different concentrations of salt solution are administered as the hydrating solution. A marked decrease in urine output is obtained when the hydrations are performed with 0.85% sodium chloride.

Adrenalectomized animals, 16 hours after adrenalectomy: Sixteen hours after adrenalectomy the animals excrete water quite slowly (Fig. 2), confirming previous reports. However, when the hydrations were performed with 0.2% saline the animals showed a much more rapid and complete excretion. Whereas animals hydrated with water excreted an average of 46% of the hydrating dose at 7 hours, similar animals hydrated with 0.2% saline excreted 74.8% of the hydrating dose. Further, the initial rate of urine ex-

cretion is much more rapid when the hydrating solution is 0.2% NaCl (Fig. 2). This effect of 0.2% sodium chloride on rate and total volume of urine is quite consistent, and the excretion is the same as that obtained in normal animals hydrated with water.

When the concentration of sodium chloride in the hydrating solution was increased the total excretion began to fall. With 0.5% sodium chloride the animals excreted an average of 66.6% of the hydrating dose at 7 hours. Although this is still definitely above animals receiving water hydration, it is lower than the excretion obtained with 0.2% sodium chloride. Thus

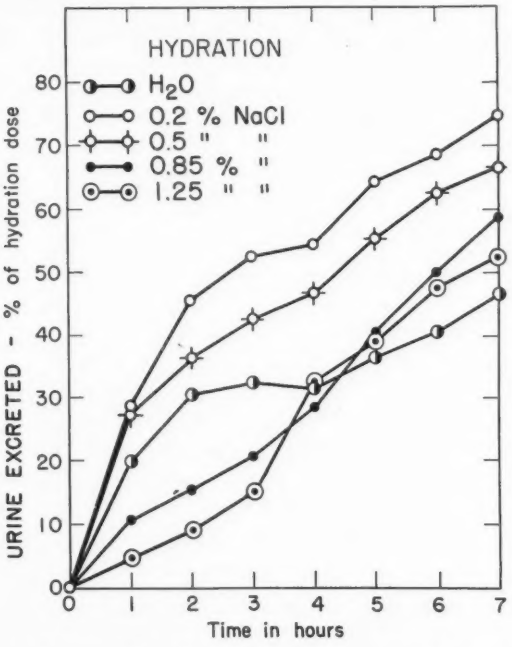

FIG. 2. Average urine excretion of rats 16 hours after adrenalectomy hydrated with water and various concentrations of saline.

the maximum effect was obtained with 0.2% sodium chloride concentration. With a further increase in sodium chloride concentration to 0.85% or 1.25% sodium chloride there is still a further decrease in the amount of the hydrating dose that is excreted.

Adrenalectomized animals, 7 days after adrenalectomy: Hydration with water 7 days after adrenalectomy gave only a very small output of urine ranging from 11 to 30% excretion in 7 hours. Hydration with 0.2% sodium chloride increased urine output but not to the degree previously observed

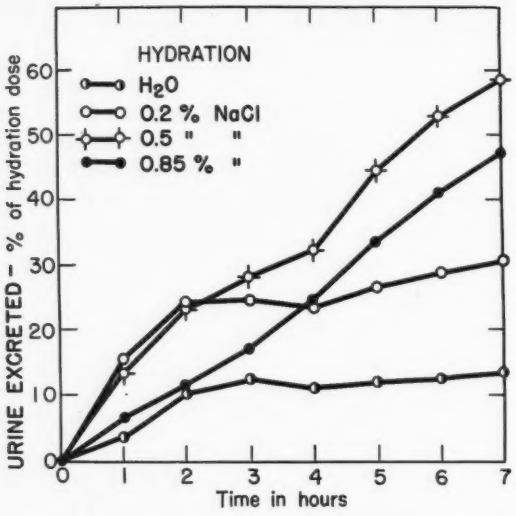

FIG. 3. Average urine excretion of rats 7 days after adrenalectomy hydrated with water and various concentrations of saline.

in animals adrenalectomized for 16 hours. However, when 0.5% saline was used as the hydrating fluid, urine excretion averaged 58.9% (Fig. 3, Table 1). This represents a high rate of excretion and it should be noted that this was achieved with a 0.5% saline solution, whereas in animals adrenalectomized for only 16 hours, the maximum effect was obtained with 0.2% saline. When 0.85% sodium chloride was used as the hydrating solution, the urine excretion again began to decrease slightly as was observed in the 16-hour adrenalectomized animals. A 1.25% sodium chloride solution was also administered. Urine excretions here are slightly higher than

TABLE 1 RELATION OF HYDRATING SOLUTION TO URINE OUTPUT OF NORMAL AND ADRENALECTOMIZED RATS

Hydrating solution	% excretion at 7th hour		
	Normal animals	Adrenalectomized	
		16 hours	7 days
H_2O	78.3 ± 3.30 (12)	46.7 ± 6.02 (16)	13.9 ± 3.06 (8)
0.2% NaCl	70.5 ± 3.14 (18)	74.8 ± 3.07 (18)	30.7 ± 3.87 (18)
0.5% NaCl	61.7 ± 3.29 (18)	66.6 ± 2.56 (16)	58.9 ± 3.58 (16)
0.85% NaCl	53.4 ± 6.88 (12)	60.9 ± 3.26 (18)	47.7 ± 3.70 (16)
1.25% NaCl	64.8 ± 2.25 (10)	52.2 ± 2.75 (16)	

Number of animals in each test listed in parentheses.

$$\text{S.E.} = \sqrt{\frac{\Sigma d^2}{n(n-1)}}$$

that obtained with isotonic saline, but are difficult to interpret because of the diarrhea that resulted from this concentration of salt in the adrenalectomized animals.

Chloride excretion: In the 16-hour and 7 day adrenalectomized rats the concentration per cc. of urine increased as the per cent of salt in the hydrating fluid was increased (Table 2). Similarly, the total urine chlorides or total urine chlorides per 100 grams of body weight also increased with the concentration of salt administered. This increase in total urine chlorides was also obtained with the 0.5% and 0.85% solutions of sodium chloride,

TABLE 2. HYDRATIONS WITH VARIOUS SALT CONCENTRATIONS AND CHLORIDE EXCRETION

	Hydrating Solution				
	H_2O	0.2% NaCl	0.5% NaCl	0.85% NaCl	1.25% NaCl
	Cl, micro eq./cc. Urine				
Normal	8.8±1.56	20.4± 1.86	75.9±3.24	168±6.8	255± 7.8
Adx, 16 hours	13.1±3.49	28.1± 1.78	78.0±2.35	159±4.0	249± 2.9
Adx, 7 days	46.8±5.49	74.1±13.6	92.2±5.57	158±8.9	212±14.4
	Cl, micro eq. total				
Normal	682±127	1.631±193	5,104±412	10,198±1,240	18,558±1,131
Adx, 16 hours	467±119	2,029±69	4,498±257	9,094±373	10,680±1,414
Adx, 7 days	613±167	1,762±194	4,953±464	6,150±28	12,021±1,838
	Cl, micro eq. total/100 gms. body wt.				
Normal	153±65.2	353±41	1,129±78	2,183±179	3,950±54
Adx, 16 hours	124±32.7	506±42	1,254±74	2,300±114	3,107±272
Adx, 7 days	183±60.2	474±74	1,334±108	1,796±232	

even though with these solutions the per cent excretion was beginning to decrease from the maximum.

Discussion: The hydration technic used provides sufficient stress to detect the inability of adrenalectomized animals to excrete orally administered water. The urine output of adrenalectomized animals 16 to 18 hours after operation is significantly below that of normal animals. A much greater impairment in renal excretion is noted 7 days after adrenalectomy. Whereas maximum urine excretion in normal animals was obtained when the hydrating solution was water the same administrations to adrenalectomized animals always produced the lowest volume of urine. Salt solutions of various concentrations administered to normal animals produce a decrease in urine output. This decrease becomes greater as the salt concentration is increased up to 0.85% saline. The reverse picture was obtained when saline solutions were administered to adrenalectomized animals. Sixteen to eighteen hours after adrenalectomy maximum urine output was obtained when the hydrating solution was 0.2% saline. These adrenalectomized animals were then able to excrete 74.8% of the administered vol-

ume by the end of the 7th hour (Table 1). This average value is similar to that obtained in normal animals hydrated with 0.2% saline. It is important in such studies to compare the effect of a given saline solution in adrenalectomized rats with the effect of the same solution in normal rats. The 0.2% sodium chloride solution which gave a maximum response to 16 to 18 hours after adrenalectomy gave only a slight effect in 7 day adrenalectomized animals. Seven days after adrenalectomy, the peak concentration of sodium chloride solution had significantly changed. The 0.5% sodium chloride produced an excretion of 58.9% in 7 days adrenalectomized animals, which was not significantly different from an excretion of 61.7% obtained with this solution in normal rats (Table 1). Therefore, even 7 days after adrenalectomy adequate urine excretion could be obtained when the proper amount of sodium chloride was administered in the hydrating solution.

If the extracellular fluid of normal dogs and rats is depleted of sodium chloride a diminished urine output will be obtained following hydration with water (Bristol, 1951). Thus, a deficiency of sodium alone in unoperated animals will markedly interfere with renal excretion. It is known that the adrenalectomized animal loses sodium and it is possible that his inability to excrete large amounts of administered water is due chiefly to a deficiency of sodium. This interpretation would be borne out by the fact that adequate urine secretion can be obtained when sodium chloride is administered with the water. It has been reported in the literature that normal (0.85%) saline is without diuretic effect in adrenalectomized animals. However, in such cases the urine output of the adrenalectomized animal hydrated with 0.85% saline is usually compared with the output obtained in normal rats hydrated with water. With the hydration doses used in the present study, the untreated adrenalectomized rat excreted 0.85% saline as well as unoperated control animals. More dilute saline solutions did, however, permit an increased urine excretion above that obtained with 0.85% saline.

The plasma level of sodium may be normal or low in adrenalectomized animals and will decrease during hydration. This change has been noted to occur even after pre-treatment with sodium chloride. It would seem important then, in such studies, that the sodium chloride be constantly available as it is when administered with the hydrating solution. It is possible that although serum sodium may be normal the reserve or total extracellular amount available may be insufficient to allow a normal rate of urine excretion, particularly when the hydrating solution is water. Further data is needed on this point. Hydration with various concentrations of sodium chloride after maintenance of the adrenalectomized animal with adrenal extract or DCA also remains to be studied.

Summary: Adrenalectomized animals hydrated with water were observed to excrete the water at a very slow rate, confirming previously published reports. However, a normal excretion may be obtained by hydrating the animals with saline solutions. The concentration of solution that gave the maximum response varied with the time after adrenalectomy. Sixteen hours after adrenalectomy a high and maximum excretion was obtained with 0.2% saline. Seven days after adrenalectomy, the maximal effective concentration had shifted from 0.2% to 0.5% saline. If the proper concentration of sodium chloride is administered with the hydrating solution, an adequate urine excretion can be obtained.

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NOTES AND COMMENTS

INHIBITION OF THE DIABETES ENHANCING EFFECT OF ACTH BY A DIET HIGH IN POTASSIUM CHLORIDE*

INTRODUCTION

It has been shown that a diet high in potassium chloride will inhibit the increase in urinary nitrogen produced by ACTH in normal rats (Whitney and Bennett, 1950). In view of this it seemed of interest to determine whether a diet high in KCl would inhibit the increase in glycosuria produced by ACTH in diabetic rats.

METHODS

All animals used were male rats of the Long-Evans strain that had an established glycosuria following partial pancreatectomy. A total of 29 diabetic rats were studied. During the period of study they were maintained in individual metabolism cages and urines were collected daily for analyses. In so far as possible, the food intake was constant for each animal throughout the period of observation. All animals were fed the stock diet and to the diet of each animal on the high KCl diet an additional 600 mg. of KCl were added. The ACTH preparation used was prepared by the method of Li, Evans, and Simpson (1943) and was injected intraperitoneally three times daily. Biological assay demonstrated that a daily dose of 0.2 mg. of these preparations maintained the adrenal weight of 40-day old hypophysectomized rats. The methods for analysis and for maintaining the constancy of the diet were the same as those previously employed (Bennett and Li, 1947).

RESULTS

In the first experiment 4 animals served as controls while 5 animals received the high KCl diet. ACTH was given at a dose level of 3 mg. per day for 3 days, then 7–8 mg. per day for 4 days. At both levels of ACTH the average glycosuria increased in both groups of animals; the average increases were 330 and 530 mg. per rat per day in the high KCl group, and 780 and 1,470 mg. per rat per day in the control group. All animals on the control diet had a statistically significant increase in glycosuria while only two of the five animals on the high KCl diet had a significant increase in glycosuria. A second identical experiment was done with another group of 9 rats. When each rat received 3 mg. per day of ACTH for 3 days, the average glycosuria in each group increased by about the same amount, approximately 1,800 mg. per rat per day. On re-examination of the data it became apparent that in the first experiment were included rats that had moderate or minimal glycosuria, excreting approximately 3,500 mg. of glucose per day or less, whereas in the second experiment the animals were more severely diabetic and none were excreting less than 3,300 mg. of glucose per day. It was only those rats with moderate or minimal glycosuria that were on the high KCl diet that failed to respond to ACTH in the usual way. In an effort to substantiate further this observation, a third experiment was done in which 6 diabetic rats were selected which had moderate to minimal glycosuria (range from traces to 2,300 mg. of glucose per day). These animals were placed on the control diet and following a 10-day control period were given increasingly large (up to 4 mg. per rat per day) doses of ACTH until an increase in glyco-

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suria was obtained. With the 4 mg. dose of ACTH the largest effect was obtained and an average increase in glycosuria of 625 mg. per rat per day was observed. An extra 600 mg. of KCl were then added to the diet of each of these animals and 8 days later an identical course of ACTH treatment from the same lot of ACTH was given. This time, less of an increase in glycosuria was observed during ACTH treatment. When the 4 mg. dose of ACTH was used the average increase in glycosuria was only 80 mg. per rat per day. A fourth experiment was done with another group of 5 moderately diabetic rats in which three courses of ACTH were given, the first and third while the animals were on the

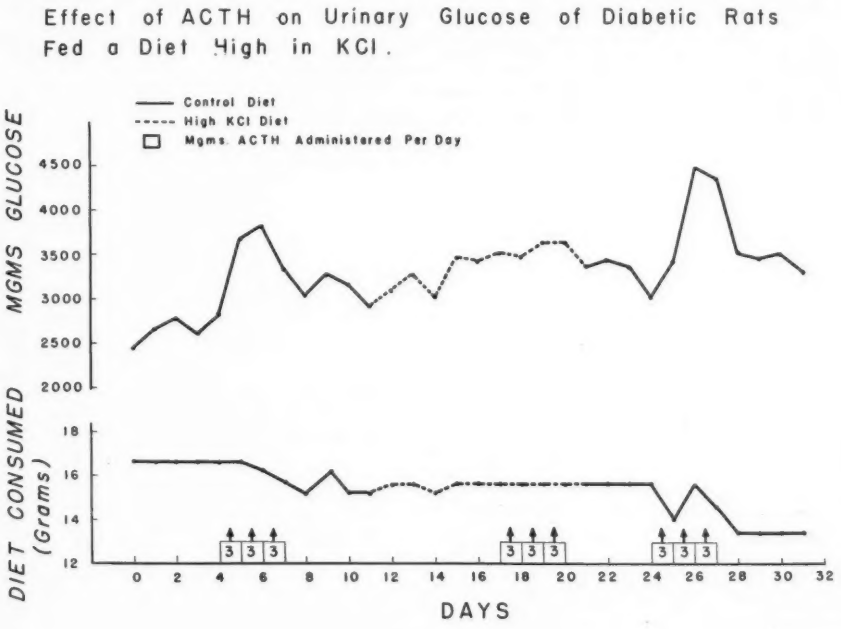

FIG. I

control diet, and the second while the animals were receiving the KCl supplement. The data from this experiment are presented in Fig. I and clearly show the absence of the diabetes enhancing effect of ACTH while the rats were on the diet high in KCl. It must be stressed that we have found this inhibitory effect of KCl only in rats with moderate or minimal glycosuria and only with doses of ACTH which are in effect minimum effective doses for increasing glycosuria.

SUMMARY

Eleven moderately diabetic rats while on the control diet exhibited an increase in glycosuria when given ACTH. When these rats were fed a diet high in KCl the same dose of ACTH had little or no effect on their glycosuria. Ten diabetic rats eating a diet high in KCl and eight diabetic rats on the control diet were given ACTH. Significant increases in glycosuria occurred in seven animals on the control diet but not in three out of four animals on the high KCl diet that were excreting less than approximately 3,500

mg. of glucose per day. It is concluded that a diet high in KCl when fed to diabetic rats with minimal or moderate glycosuria inhibits the increase in glycosuria produced by minimum effective doses of ACTH.

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